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NOTE ON THE PRESENCE OF IODINE IN LARGE QUANTITIES OF SHEEP PITUITARY GLAND.

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Because of the similarity between the pituitary and the thyroid glands in their physiological action as well as their embryological history, the question as to whether the pituitary gland contains any iodine, the element generally conceded to be vitally important in the functioning of the thyroid, is still an important one.

In 1896 Baumann (1) examined human pituitaries for the presence of iodine always with negative results. Schnitzler (2) also examined human pituitary glands using larger amounts of the material and found iodine twice. In 1909 Wells (3) analyzed human pituitaries and recovered definite amounts of iodine. In 1911 Denis (4) examined human pituitary glands and found no iodine.

Wells suggested in his paper that his ability to find iodine in the glands he examined might have been due to the fact that iodine had previously been used on these patients either as medicants or in dressings and that the element might have been absorbed by the gland from these external sources.

Denis made a careful study of the history of the cases whose glands she examined and excluded the possibility of iodine having been used in any way. She was not able to detect any trace of the element.

The largest amount of material examined was by Schnitzler when he analyzed 19 and 24 gm. of pituitary. In both cases he found iodine. It has been suggested that the iodine may exist in the pituitary gland in such infinitesimally small amounts that only by the use of large quantities of material could it be detected. Therefore, an attempt was made in this laboratory to examine pituitary glands in larger amounts than had previously been used.

Fresh sheep pituitary glands were obtained, trimmed, ground, dried, and powdered. The presence of iodine was determined by the Baumann-Riggs method. Three samples were examined, the first consisting approximately of 30 gm.; the second 50 gm.; and the third 100 gm. In none of these samples was any iodine detected. These findings corroborate the findings of Denis.

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1. Baumann, E., *Münch. med. Woch.*, 1896, xliii, 309.
2. Schnitzler, J., *Wien. klin. Woch.*, 1896, ix, 657.
3. Wells, H. G., *J. Biol. Chem.*, 1909-10, vii, 259.
4. Denis, W., *J. Biol. Chem.*, 1911, ix, 363.

HEMATO-RESPIRATORY FUNCTIONS.*

III. THE FALLACY OF ASPHYXIAL ACIDOSIS.

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One of the ideas basic in the physiology of today is that a deficiency of oxygen produces in the blood a slight, but in its effects highly potent, acidity. Araki[2] observed that partial asphyxia causes the appearance of lactic acid (lactates) in the urine, and this has been interpreted to indicate that, in the absence of an adequate supply of oxygen, a condition which would now be called acidosis tends to develop.

This interpretation of Araki's observation has gained support from the fact, now apparently well demonstrated, that the first step chemically in the production of energy in the animal body is anaerobic: sugar breaking down into lactic acid, which, as a second step and as a process of elimination rather than of energy production, is then oxidized to carbon dioxide. The increase of lactic acid in blood and urine after vigorous muscular work, as found by Ryffel,[3] is thus apparently easily explained.

The hypothesis of asphyxial acidosis has seemed to fit in well also with the regulation of respiration by the chemical condition of the blood; for if the hormone of breathing is the CH of the blood, an increased production, or delayed destruction, of a strong

* This and the following papers are continuations of work published by us in this *Journal* some months ago under the same general title.[1] All these investigations were performed at about the same time (1918) and under the same conditions and collaboration.

[1] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxix, 163.

[2] Araki, T., *Z. physiol. Chem.*, 1894, xix, 422.

[3] Ryffel, J. H., *J. Physiol.*, 1909–10, xxxix, p. xxix.

organic acid would at first sight appear to afford the conditions for the increased volume of breathing caused by asphyxia, and for the overbreathing and lowering of the alveolar CO_2 induced by intense muscular exertion.[4]

On the other hand, doubt should have been aroused by the fact that in non-vital chemistry it is oxidation which tends to produce acids while deoxidation usually has the contrary effect. Macleod and Knapp[5] have found that the intravenous administration of alkali (Na_2CO_3) causes an increase of lactic acid (lactates) in the blood. This is important, for if this occurs as the result of alkalosis the appearance of lactates in the urine under the little understood conditions of asphyxia and of muscular exertion can scarcely be taken as a certain indication of acidosis. Indeed, when we examine the current conception critically and quantitatively, there appears to be very little ground for the belief that lactic acid is ever a really important factor in increase of breathing.

The chief supporter of the idea that a production of lactic acid is the cause of hyperpnea has been Haldane. About a year ago we published our dissent from this view.[6] A reprint, which we forwarded to Dr. Haldane, reached him just as he was sending to press a paper,[7] in which he discards his previous opinion and concurs with us in recognizing that under partial asphyxia the blood is altered, not toward acidosis, but in reality, because of overbreathing, toward alkalosis.

Indeed there seem to be signs of a drift away from the crude idea of the influence of acids in the body which has prevailed for some years, and which a too mechanical application to physiology of the physicochemical conception of the regulation of CH has for a time exacerbated rather than corrected. But a broader view is coming now. It is beginning to be recognized, for instance, that the symptoms of the so called acidosis of diabetes are due

[4] Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1908–09, xxxviii, 420–440.

[5] Macleod, J. J. R., and Knapp, H. J., *Am. J. Physiol.*, 1918–19, xlvii, 189.

[6] Henderson, Y., *Science*, 1919, xlix, 431.

[7] Haldane, J. S., Kellas, A. M., and Kennaway, E. L., *J. Physiol.*, 1919–20, liii, 181.

to the acetone derivatives as such, and not to their nature as acids. The fallacy of asphyxial acidosis is, however, deeply ingrained in current thought and will probably be removed with difficulty. Thus, nearly all the investigators who have worked recently, for instance, on the shock problem have accepted explicitly the view, stated by Bayliss,[8] that "when the blood pressure is allowed to remain at a low level for some considerable time, there is a decrease of the bicarbonate concentration of the blood, owing to the production of some fixed acid by the tissues when inadequately supplied with oxygen." In reality, the fatal lowering of the blood alkali after hemorrhage is due, as we shall show in a later paper, to a process of an altogether different nature; namely, overbreathing similar to that produced by oxygen deficiency. The observations of Henderson, Haggard, and Coburn[9]—now in practical clinical use—have demonstrated the beneficial effects of inhalation of 6 or 8 per cent of CO_2 in air in the closely related form of surgical depression induced by prolonged and extensive major operations without hemorrhage. They find that such inhalations rapidly recall a normal alkali content to the blood, with a corresponding restoration of the circulation and other functions. If the view above quoted from Bayliss were correct, such treatment would kill instead of cure.

Inconsistency of the Present Theory.

For the sake of testing the current hypothesis of asphyxial acidosis, let us suppose that under oxygen deficiency lactic and similar acids are thrown into the blood. The results logically to be expected from their action are not at all those which experiments on partial asphyxia actually afford.[10] Such an experiment has been performed many hundreds of times in the test for aviators introduced by one of us[6, 11] into the United States Army

[8] Bayliss, W. M., *J. Pharmacol. and Exp. Therap.*, 1920, xv, 64.

[9] Henderson, Y., Haggard, H. W., and Coburn, R. C., *J. Am. Med. Assn.*, 1920, lxxiv, 783.

[10] Henderson, Y., *Am. J. Physiol.* 1909–10, xxv, p. xii. Henderson, Y., and Scarbrough, M. M., *Am. J. Physiol.*, 1910, xxvi, 271.

[11] Henderson, Y., Seibert, E. G., Schneider, E. C., Whitney, J. L., Dunlap, K., Wilmer, W. H., and Berens, C., Lewis, E. R., and Paton, S., *J. Am. Med. Assn.*, 1918, lxxi, 1382–1400.

during the war. It consists in rebreathing a small volume of air (50 to 100 liters) and thus reducing its oxygen tension, while the CO_2 exhaled is absorbed by a cartridge of caustic alkali. With this and similar apparatus the careful observations of Lutz and Schneider[12] have shown that good candidates for high flying develop a progressive increase in the volume of breathing beginning even with a slight reduction (2 or 3 per cent of an atmosphere) in the tension of oxygen in the air inhaled.

On the assumption of the hypothesis that lactic acid is being produced in the bodies of these men and is escaping oxidation the following facts must be borne in mind.

1. 1 molecule of lactic acid normally burns to 3 of CO_2.

2. Any reasonable amount of lactic acid entering the blood reacts immediately, and practically completely with $NaHCO_3$. Each molecule of the acid liberates 1 molecule of CO_2. As the amount of breathing is determined by the amount of CO_2 requiring to be exhaled, the first effect of decreased oxidation of lactic acid should be, therefore, to decrease respiration in the ratio 1:3 for whatever fraction of the lactic acid escapes combustion.

3. At the same time, however, the blood alkali is decreased, with formation of sodium lactate; and, as we have shown,[1] the volume of breathing increases in inverse proportion to the blood alkali. If, then, we calculate the percentage increase of breathing which should ultimately result from a neutralization of a part of the $NaHCO_3$, and the amount of decrease of breathing which would be caused temporarily by the lessened CO_2 production in the body from non-combustion of the lactic acid, we get some such result as the following:

A reduction of blood alkali of, for instance, 10 volumes per cent involves an increase in the volume of breathing per unit mass of CO_2 eliminated of roughly 20 per cent. Thus a man who previously breathed 8 liters of air per minute would now breathe 9.6 liters. If the whole CO_2 capacity of the blood in the body is taken as 2,500 cc., a lowering of alkaline reserve from 50 volumes per cent to 40 would require enough lactic acid to liberate 500 cc. of CO_2. Thus the CO_2 exhaled would be reduced by 1,000 cc. and the volume of air would be diminished by 25 liters (since each

[12] Lutz, B. R., and Schneider, E. C., *Am. J. Physiol.*, 1919, l, 280.

liter of air breathed removes about 40 cc. of CO_2) before the neutralization of lactic acid and decrease of $NaHCO_3$ in the blood would balance the respiratory effect. Until this occurred the breathing would be markedly subnormal.

The fact is, on the contrary, that under oxygen deficiency there is an almost immediate slight increase of breathing, as Lutz and Schneider[12] have shown.

Furthermore, the theory of asphyxial acidosis requires that there should be, prior to or at least coincident with the increase of breathing, a lowering of the blood alkali as measured by its CO_2-combining power. But this deduction likewise fails to check with the facts. For even after 60 or 90 minutes of marked oxygen deficiency the blood alkali shows only a slight fall. (A large fall would undoubtedly occur in the course of hours—that is, as acclimatization develops—but this is another matter and will be dealt with in succeeding papers.)

Immediate Effect of Diminished Oxygen on the Breathing and Blood Alkali.

In support of these statements we give the observations which follow—a line of work initiated by one of us while directing the physiological investigations at the Medical Research (Aviation) Laboratory at Mineola and continued there by Professor Schneider and Dr. Lutz, to whom we are indebted for the opportunity to quote the data here.

A sample of blood was taken, equilibrated with air containing CO_2 at 40 mm. tension, and its CO_2-combining power determined by analysis. The subjects, healthy men, then entered a steel chamber; the air pressure was pumped down to the equivalent of an altitude of 15,000 or 18,000 feet in 15 or 18 minutes, and was maintained at this level for about $1\frac{1}{4}$ hours. Another blood sample was then taken, either shortly before or soon after the normal barometric pressure was restored. Observations were made on the volume of breathing, either by direct measurement or indirectly through analyses of the alveolar air. The data are given in Table I.

From these data it appears that deficiency of oxygen may induce a marked increase of breathing long before a perceptible

TABLE I.

Showing Effects on Healthy Men of Diminished Oxygen in Increasing Volume of Breathing before Any Considerable Lowering of Blood Alkali Occurs.

Experiment No.	Time.	Barometer.	Alveolar air.		Volume of breathing.		Blood CO_2 capacity.
			O_2	CO_2			
	min.	*mm.*	*mm.*	*mm.*	*liters per min.*	*per cent of normal*	*vol. per cent*
I	0	760	106	39		100	51
	18	380	41	30		130	
	92	380	38	34		115	
	98	380					48
II	0	760	105	37		100	52
	20	428	41	33		112	
	93	428	49	26		142	
	111	760	105	35		106	49
III	0	760	99	37		100	58
	19	380	42	32		115	
	73	380	36	31		119	
	99	760	98	40		92	57
IV	0	760	93	45		100	55
	18	380	63	29		155	
	53	380	47	24		187	56
	71	760	81	40		112	
V	0	760	101	43		100	49
	18	380	31	35		123	
	85	380	34	31		139	
	90	380					49
	111	760	108	35		123	
VI	0	760	99	40		100	
	48	380	31	34		118	
	89	380	33	29		138	
	112	760	97	36		111	
VII	0	760			7.2	100	61
	21	380			9.3	129	
	90	380			7.6	105	
	94	380					65

TABLE I—*Concluded.*

Experiment No.	Time.	Barometer.	Alveolar air.		Volume of breathing.		Blood CO_2 capacity.
			O_2	CO_2			
	min.	*mm.*	*mm.*	*mm.*	*liters per min.*	*per cent of normal*	*vol. per cent*
VIII	0	760			5.5	100	57
	18	380			7.1	129	
	80	380			5.7	103	57
IX	0	760			6.2	100	61
	20	380			6.6	106	
	41	380			7.4	119	
	68	380			7.3	118	61

reduction of the blood alkali has occurred, and at first out of all proportion to the reduction. Neutralization of blood alkali by lactic acid cannot, therefore, be the causal factor in producing the initial hyperpnea of oxygen deficiency. (We shall show in the next paper[13] that a continuation for a longer time of overbreathing and blowing off of CO_2 results in a compensatory disappearance of alkali from the blood—an adjustment which tends ultimately to restore the normal ratio of $H_2CO_3 : NaHCO_3$. The process involved is, however, exactly the opposite of that demanded by the theory of asphyxial acidosis.)

Effects of Intravenous Injection of Lactic Acid.

The nearest experimental imitation of the assumed flooding of the circulation with lactic acid under oxygen deficiency, or during muscular work, is afforded by injecting lactic acid into the blood. We have tried this on dogs and have compared the effects with those obtained in a series of experiments, already published by us,[1] in which normal hydrochloric acid was injected, and a true and intense acidosis obtained. It will be seen that the experiments with lactic acid differ markedly from those with hydrochloric.

Protocol 1.—Dog, male, 8 kilos. Normal lactic acid (9 per cent) was administered intravenously, 5 cc. at a time, to a total of 140 cc., in the course of 3 hours. At the end of this time the animal died.

[13] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920, xliii, 15.

Each injection caused a temporary increase of breathing. The respiratory volume at these times, however, never exceeded 3.0 liters of air per minute, while the normal breathing between injections averaged 2.0 liters at first and increased gradually to 2.5 liters.

Toward the end of the experiment the animal voided a red urine which showed on microscopic examination a large number of erythrocytes. Autopsy revealed the heart in diastole, the kidneys acutely inflamed, and the bladder filled with bloody urine.

Samples of blood were drawn from the femoral artery at the beginning and near the end of the experiment, were equilibrated with air containing 5.5 per cent CO_2, and then analyzed. The CO_2 capacities thus found were as follows:

	vol. per cent
CO_2 before injection of lactic acid	45
CO_2 after " " " "	39
CO_2 decrease	6

Protocol 2.—Dog, female, 6 kilos. Normal lactic acid was injected into the jugular vein at the rate of 3 cc. per minute. The animal exhibited a moderate respiratory augmentation. It urinated profusely during the last 10 minutes of the experiment, and died quite suddenly after 32 minutes, when 90 cc. of acid had been injected. The blood was found to be very dark in color and on standing a reddish plasma separated.

The CO_2 capacity of the blood was determined as in the previous experiment, and showed:

	vol. per cent
CO_2 before injection of lactic acid	42
CO_2 after " " " "	39
CO_2 decrease	3

Three points stand out in these experiments, when they are compared with those performed with hydrochloric acid.

1. It takes from two to three times as much lactic acid to kill a dog as it does, molecule for molecule, with hydrochloric acid.

2. Whereas with the smaller amounts of hydrochloric acid the blood alkali was reduced in many cases to half the normal value, the reduction induced by lactic acid was comparatively slight.

3. Animals poisoned with hydrochloric acid become truly acidotic and die in convulsions, but those killed by means of lactic acid die in quite a different manner, apparently by heart failure.

We are left with the strong impression that lactic acid, even the optically inactive form used in these experiments, is rapidly destroyed, and that the death of these animals was due to the injury to the heart and kidneys induced by the too rapid injection

of the substance. Short of such acute effects it appears from these experiments that no considerable degree of acidosis can be induced with lactic acid.

Cause of Overbreathing.

What, then, is the causal connection between deficiency of oxygen, or intense muscular exertion, and overbreathing? We suggest that in the present state of knowledge it may be appropriately denominated as respiratory *x*.

While it is not possible to name this important factor in breathing more exactly, the following statements may be made regarding its character and properties.

It is not a strong, fixed acid. If it has any acid property it must be extremely weak. It tends not to raise C_H, but through overbreathing, to lower it. The volume of breathing varies as the sum, or product, of respiratory *x* and C_H.[14] (Strictly speaking, when we write C_H, we mean the CO_2 ratio.)

It acts, in large amounts, very much as does ethyl ether in the excitement stage of anesthesia. Its later effects resemble the disagreeable manifestations of alcoholic intoxication. But it may be quite a different type of substance from the ethereal substances thus suggested.

It is produced in active muscles, and is carried to the respiratory center in the blood.[15]

It is antithetic in its respiratory effects to morphine.

It may be a substance, or substances, or it may be an altered state of some blood constituent or structure; *e.g.*, the permeability of the surface of the red corpuscles to certain ions, or liberated hemoglobin.

Paradox of Anoxemic Hyperpnea.

Finally, we may here call attention to the discrepancy between theory and fact, and between two phases of present theory, in respect to the interaction of CO_2 and oxygen upon the C_H of the

[14] Hasselbalch, K. A., and Lundsgaard, C., *Skand. Arch. Physiol.*, 1912, xxvii, 13.

[15] Geppert, J., and Zuntz, N., *Arch. ges. Physiol.*, 1888, xlii, 189.

blood on the one hand, and in respect to the influence of the C_H of the blood upon the respiratory center on the other.

Several writers, Christiansen, Douglas, and Haldane, Parsons, L. J. Henderson,[16] and others, hold that oxygenation of the blood increases the acid property of hemoglobin and assists in driving out CO_2, and that deoxygenation of the blood should lower C_H and lower the CO_2 tension at a uniform CO_2 content. As the respiratory center is acutely sensitive to alterations of C_H, the inhalation of nearly pure nitrogen or hydrogen by lowering the C_H of the blood should therefore depress respiration. In fact, however, acute oxygen deficiency for even a few seconds induces hyperpnea in most persons (*cf.* Lutz and Schneider[12]).

This paradox appears at present insoluble. To say merely that oxygen deficiency is itself a stimulus[17] or that it governs the excitability of the respiratory center for CO_2[18] leaves the question as to how it does this quite unanswered. But evidently the stimulation of breathing in this case is not due to increase of C_H, for C_H is altered in one sense while the activity of the respiratory center is altered in the physiologically opposite sense.

As expressed by Hasselbalch and Lungsgaard,[14] the responsiveness of the respiratory center to the stimulus of CO_2 or C_H varies inversely with the oxygen tension. A somewhat closer statement of these relations seems to us to be as follows: respiratory x is increased, the responsiveness of the respiratory center to C_H intensified, and the breathing thereby augmented, so that the ratio H_2CO_3 : $NaHCO_3$ is reduced, whenever the ratio of the oxygen tension in the lungs (in mm.) to the alkali in use in the arterial blood (measured in volumes per cent of CO_2 capacity) is considerably reduced below the normal value. When (as after descending from a great altitude or in some phases of nephritis) the oxygen: alkali ratio is above normal, respiration is relatively depressed. Thus acidosis[1] (high ratio of H_2CO_3 : $NaHCO_3$) is induced.

[16] Christiansen, J., Douglas, C. G., and Haldane, J. S., *J. Physiol.*, 1914, xlviii, 244. Parsons, T. R., *J. Physiol.*, 1919–20, liii, 42; Henderson, L. J., *J. Biol. Chem.*, 1920, xli, 401.

[17] Gasser, H. S., and Loevenhart, A. S., *J. Pharmacol. and Exp. Therap.*, 1913–14, v, 239.

[18] Lindhard, J., *J. Physiol.*, 1911, xlii, 337.

CONCLUSION.

The prevailing conception[19] of asphyxial acidosis is in many respects diametrically opposed to the facts. Under low oxygen overbreathing occurs before the blood alkali is appreciably reduced.

Intravenous injections of lactic acid do not induce an acidosis at all commensurable with the amount administered. It is improbable that such a condition as "lactic acid acidosis" ever occurs in life. An increase of lactates in the blood or urine is probably an indication, not of acidosis, but of low ratio of H_2CO_3: $NaHCO_3$; that is, alkalosis.

It is suggested that the stimulant produced under low oxygen be termed respiratory x. The only fact certainly demonstrable about it is that it is not a strong, fixed acid. It causes, not acidosis, but overbreathing and alkalosis.

In the next paper[13] we shall deal with the character of the reaction which the body makes to oxygen deficiency.

[19] For a general presentation of the present conception of acidosis see the discussion by Henderson, L. J., Howland, J., Woodyatt, R. T., Frothingham, C., Rowntree, L. G., Henderson, Y., and Van Slyke, D. D., *New York Med. J.*, 1916, civ, 1119.

HEMATO-RESPIRATORY FUNCTIONS.

IV. HOW OXYGEN DEFICIENCY LOWERS THE BLOOD ALKALI.

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The theory that under oxygen deficiency a production of acid occurs in the body with partial neutralization of the blood alkali has been shown in the preceding paper[1] to be no longer tenable as an explanation of anoxemic hyperpnea. It has prevailed heretofore largely because of the lack of an alternative explanation.

The problem is one which bears upon many and diverse conditions; upon diabetic and some other forms of hyperpnea, upon conditions in disease in which the oxygenation of the blood in the lungs is diminished, upon the depression of vitality and lowering of the blood alkali during and after ether anesthesia,[2] upon overbreathing from muscular exertion, upon acclimatization to various altitudes above sea level, and upon the ability of aviators to withstand low barometric pressures.[3]

As an alternative to oxygen deficiency in the causation of mountain sickness, Mosso[4] suggested that an abnormally rapid volatilization of CO_2 out of the blood may occur under reduced barometric pressure. He termed this acapnia. The idea has in it a germ of truth, but as formulated by Mosso is quite untenable. We have recently shown, however, that the body is

[1] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920, xliii, 3. Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1920, xli, p. xlv.

[2] Henderson, Y., Haggard, H. W., and Coburn, R. C., *J. Am. Med. Assn.*, 1920, lxxiv, 783.

[3] Henderson, Y., *Science*, 1919, xlix, 431.

[4] Mosso, A., *Arch. ital. biol.* (numerous papers), 1903–05, xxxix, xli, xlii, xliii.

capable of a reaction[5] which affords an explanation in the direction in which Mosso pointed. The whole matter turns upon the process through which the blood alkali is decreased under low oxygen.

According to the now current conception, the ionic balance of acids and bases in the blood depends upon the maintenance of a certain ratio (about 3:60) between the dissolved CO_2 and the alkali bicarbonates; or, as expressed in the well known equation of L. J. Henderson,[6] $\frac{H_2CO_3}{NaHCO_3} \times K = C_H$. (We shall here assume that in this equation K, which we have termed the dissociation characteristic, is absolutely constant. If it should be shown that this factor also varies, the proportionality of C_H to the ratio H_2CO_3:$NaHCO_3$ will require a corresponding correction.)

The respiration of a healthy man ordinarily maintains in the blood a certain definite value of C_H which is probably almost the same[7] when he is acclimatized to sea level or to an altitude of 5,000 or 10,000 or perhaps even 15,000 feet above sea level. At these altitudes, however, he breathes quite different volumes of air per unit mass of CO_2 eliminated. Thus, as was shown by the Pike's Peak expedition,[8] a man maintains tensions of about 39 mm. of CO_2 in the alveolar air at sea level and only 26 mm. at 14,000 feet, for evidently the more air he breathes the more the CO_2 in the lungs is diluted. As the H_2CO_3 of the blood is determined through direct physical action by the tension of CO_2, it follows that in all conditions of equilibrium, that is in complete acclimatization to any altitude, the volume of air breathed must be adjusted to an inverse proportion with the amount of alkali in use in the arterial blood. In this way only can the ratio H_2CO_3: $NaHCO_3$ be maintained alike when the figures are 3:60, or 4:80, or 2:40, or any intermediate values—all of which give the normal ratio of 5 per cent; *i.e.*, dissolved CO_2: combined CO_2 :: 3:60 :: 5:100. Evidently this ratio may be normal at nearly any amount of blood alkali, and it may be altered or

[5] Henderson, Y., and Haggard, H. W., *J. Biol. Chem.*, 1918, xxxiii, 333, 345, 355, 365.

[6] Henderson, L. J., *Ergebn. Physiol.*, 1909, viii, 254.

[7] Hasselbalch, K. A., and Lindhard, J., *Biochem. Z.*, 1915, lxviii, 265, 295.

[8] Douglas, C. G., Haldane, J. S., Henderson, Y., and Schneider, E. C., *Phil. Tr. Roy. Soc. London, Series B*, 1912, ccii, 302.

restored by change in either member of the ratio. In the body, as we shall have occasion to show, either member follows the other back toward the normal relation.

(We suggest that the term CO_2 ratio be used to replace "H_2CO_3: $NaHCO_3$," "ratio of dissolved CO_2 to combined CO_2," and "relation, proportionality, or ratio of alveolar CO_2 tension to alkaline reserve," and that it be expressed as a percentage, as above.)

Evidently if acid is added to the blood, or alkali withdrawn, there will be two forms of augmentation of breathing: first, a temporary augmentation to remove the excess of CO_2 liberated; and second, the maintenance of the larger volume of breathing necessary to keep the tension of CO_2 in the lung air at the lower value to correspond to the diminished blood alkali, and keep the CO_2 ratio normal. Such diminution of alkali and its consequences may be termed the acidotic process. It involves, during its development, a high CO_2 ratio. We have not denied that such a process occurs. We have ourselves shown that increase of breathing may be induced by intravenous injection of acid. Our point is merely that under low oxygen or muscular exertion, and probably under other conditions, the overbreathing is so great that the CO_2 ratio is reduced below normal, and the C_H of the blood varies toward the alkaline, instead of the acid, side of neutrality.

In contrast to the acidotic process the body is endowed, as we have shown,[5] with the capacity to bring the CO_2 ratio (*i.e.*, H_2CO_3 : $NaHCO_3$) back to normal in another way, as follows: whenever respiration is excited to such activity that the H_2CO_3 of the blood is reduced below its normal relation to the alkali, a compensatory fall of $NaHCO_3$ follows, either through a passage of alkali out of the blood into the urine and tissues, or by immobilization within the blood itself, or through some equivalent readjustment. This is the acapnial process—the reaction to an alkalosis due to a relative deficiency of CO_2.

Both the acidotic and the acapnial processes result in a lowered alkali, but otherwise they are fundamentally different both in their intermediate and their terminal states. During the acidotic process the CO_2 ratio is above normal and there is therefore a high C_H, or acidosis. During the acapnial process on the other hand, the CO_2 ratio (H_2CO_3 : $NaHCO_3$) is below normal. There

is a low C_H and a state of alkalosis. Both result in hypocapnia or low alkali carbonate in the blood.

Differentiation of the Acidotic and the Acapnial Processes.

In attempting to decide which of these conditions occurs as the result of deficiency of oxygen two facts regarding the influence of the oxygen supply upon the volume of breathing must be kept in mind: (*a*) decrease of oxygen tension, even slight, induces temporarily[9] overbreathing and blowing off of CO_2; and (*b*) the readjustment to decreased oxygen tension, or acclimatization to altitude, involves (as was shown by the Pike's Peak expedition[8]) a maintained increase of breathing and a normally lower tension of CO_2 in the air of the lungs—26 mm. in contrast to 39 mm. at sea level.

From gross appearances these phenomena could be, and until the present time have been, explained as due to the acidotic mechanism. We propose here to show that, on the contrary, deficiency of oxygen and its product, the stimulant "respiratory *x*,"[1] act, not through a neutralization of blood alkali, but through overbreathing, alkalosis, and the acapnial process.

To this end we shall make use in presenting our experiments of the CO_2 diagram used in our earlier work on the effects of intravenous injection of hydrochloric acid.[10] The full force of the evidence can be seen only by comparing the two sets of experiments, those with hydrochloric acid and those under low oxygen. The gist of the matter is as follows:

In the CO_2 diagram one coordinate expresses the tension of CO_2 in the air in the lungs and in the arterial blood, while the other shows the alkali in use and the CO_2-combining power of the blood. A diagonal line, the *OC* line, in which all points have the same ratio of abscissa : ordinate, indicates therefore the normal ratio of H_2CO_3 : $NaHCO_3$; *i.e.*, the CO_2 ratio and (presumably) the C_H. The CO_2 dissociation curve of the blood is plotted from analyses of the blood at tensions of 18, 40, and 72 mm. at body temperature. The position at which the arterial blood comes on

[9] Lutz, B. R., and Schneider, E. C., *Am. J. Physiol.*, 1919, l, 280.

[10] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxix, 163.

this curve is found either by analyses of the CO_2 content of the blood, or of the CO_2 tension of the pulmonary air.

If then the arterial point falls to the right of the *OC* line there is an abnormally high CO_2 ratio ($H_2CO_3 > NaHCO_3$), high C_H, and acidosis. But if it falls to the left of the line it indicates overbreathing, lowered CO_2 ratio, reduced C_H, and, in other words, alkalosis.

It will be seen in the experiments reported here that it is the latter condition which occurs under low oxygen, and that in the course of hours the CO_2 dissociation curve, that is the blood alkali, falls lower and lower, indicating the compensatory passage of alkali out of the blood. Thus the point *A*, indicating the arterial blood, descends to the left of the *OC* line. This contrasts sharply with the process seen in our previous experiments under injection of hydrochloric acid in which *A* descended to the right of the *OC* line. It is noteworthy, however, that in the return from low oxygen to atmospheric air (in Experiments 2 and 3) respiration is depressed—not perhaps absolutely, but relatively to the blood alkali. Thus during recovery the CO_2 ratio rises above normal, the arterial point swings to the right of the *OC* line, and the rise of the dissociation curve indicates that the result of this acidosis is to recall alkali to the blood.

EXPERIMENTAL.

Our experiments were performed upon dogs. They received no general anesthetic, but were handled gently and firmly, so that no disturbance of respiration, a sure sign of physical or mental discomfort, occurred. Blood samples were obtained from the femoral artery, exposed under cocaine.

The essential experimental condition was that of rebreathing the air in a large spirometer through alkali. The animals thus gradually consumed the oxygen of the air, and developed anoxemia. This also involves no discomfort, as we know from extensive experience on men, even when the asphyxia is pushed to the point of unconsciousness.

A conical mask of sheet zinc was fitted over the head up to the eyes and made tight with adhesive plaster with the greatest care to prevent leaks. It was connected with as little dead space as

possible to inspiratory and expiratory valves. Through one of these valves the animal inhaled from a spirometer of 200 liters capacity; through the other the expired air passed by way of a cartridge containing 2 liters of finely shaved NaOH back into the spirometer. The air in the spirometer was analyzed for its oxygen content and to insure the absence of more than a trace of CO_2.

A gas meter of low resistance could be inserted temporarily in the circuit; and the volume of breathing was thus measured at intervals.

In one experiment the gaseous conditions of the interior of the body were followed by means of the "abdominal air method."[11] 2 liters of a gas mixture of 7 per cent oxygen and 93 per cent nitrogen were introduced into the peritoneal cavity before the beginning of the experiment and samples were withdrawn and analyzed at intervals.

Experiment 1.—Dog, female, 7.5 kilos. Rebreathed from spirometer containing 200 liters of air beginning at 12 m. Progressive decrease of oxygen tension in the inspired air until death, 7 hours later. Columns 4, 5, and 6 show the data from which the CO_2 dissociation curves, numbered as in Column 1, are plotted in Fig. 1. Column 8 shows the H_2CO_3 in the arterial blood, and Column 9 the $NaHCO_3$ in use. Column 10 gives the CO_2 ratio (H_2CO_3:$NaHCO_3$) and Column 11 the C_H calculated from this ratio. The abdominal air and volume of respiration were determined as described in the text.

Curve No.	Time.	Oxygen tension.	Blood equilibrated with CO_2 at			Arterial blood.			CO_2 ratio.	C_H 7 (Char. 8).	Abdominal air.		Volume of respiration.
			18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.			O_2	CO_2	
		mm.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*		*mm.*	*mm.*	*liters per min.*
1	11.50 a.m.	145	29	44	54	44	2.70	41.30	6.54	0.523	56	40	3.1
2	1.55 p.m.	118	29	44	55	44	2.70	41.30	6.54	0.523	54	40	3.1
3	5.40 "	55	29	42	51	40	2.40	37.60	6.39	0.511	38	36	4.4
4	6.20 "	30	22	36	45	26	1.37	24.63	6.10	0.498		20	10.1
5	7.00 "	22	21	33	42	21	1.14	18.86	6.04	0.483	30	17	12.0
6	7.20 "	21	17	23	29	14	0.74	13.26	5.59	0.446			
	7.22 "	Death.											

[11] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxviii, 71.

Note that the increase of breathing and lowering of arterial CO_2 content exceed the degree of reduction of alkali, as indicated by the combined CO_2, and that the CO_2 ratio and C_H are therefore lowered. The causal sequence evidently is (1) lowering of oxygen tension, (2) an increased production of respiratory x, (3) excessive respiration and a blowing off of CO_2, (4) decrease of the CO_2 ratio and lowering of C_H, that is alkalosis, and (5) the compensatory disappearance of alkali from the blood.

Experiment 2.—Dog, male, 12 kilos. Breathed an atmosphere of progressively decreasing oxygen tension from 12 to 5.44 p.m.; thereafter normal air.

Curve No.	Time.	Oxygen tension.	Blood equilibrated with CO_2 at			Arterial blood.			CO_2 ratio.	C_H[7] (Char. 8).
			18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		mm.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	
1	11.45 a.m.	155	38	49	60	51	2.96	48.04	6.18	0.493
2	3.55 p.m.	70	30	44	55	38	2.08	35.96	5.78	0.462
3	5.30 "	30	27	38	47	29	1.48	27.52	5.38	0.430
4	6.35 "	155	32	44	55	44	2.70	41.30	6.54	0.523

Note that the development of alkalosis, lowered CO_2 ratio, and lowered C_H in Columns 10 and 11 is due to overbreathing. This is indicated by the fact that the arterial CO_2 content is reduced much more rapidly than the CO_2 capacity. Note that in Columns 4, 5, and 6 the blood alkali or CO_2-combining power falls, but this fall is much slower than that of the arterial CO_2 content. Note that after restoration to the normal air the ratio $H_2CO_3 : NaHCO_3$ (Column 10) and the C_H rise above normal (due to depressed breathing), and alkali is gradually recalled to the blood. Figures in Column 1 refer to the curves plotted in Fig. 2.

Experiment 3.—Dog, male, 12.4 kilos. Rebreathed an atmosphere of progressively decreasing oxygen tension from 12 to 4.10 p.m.; thereafter normal air. At 4.10 p.m. the animal was unconscious and apparently

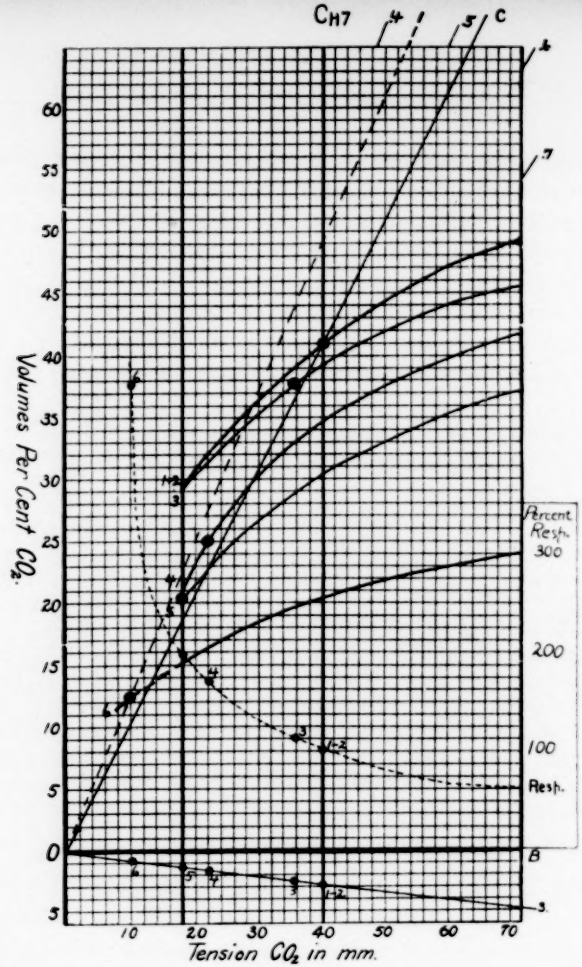

FIG. 1. The CO_2 diagram of the blood of a dog under progressively lower tensions of oxygen. From the data of Experiment 1.

OS shows the CO_2 dissolved at all tensions of CO_2. It is positive, not negative. The CO_2 dissociation curves 1 to 6 are plotted from analyses of the blood equilibrated to tensions of 40, 72, and 18 mm. (in this order) at body temperature. The dots indicate the arterial blood. The *OC* line shows the normal CO_2 ratio and (presumably) the C_H of the blood. Deviation of the arterial point to the left of the *OC* line indicates overbreathing, low alveolar and arterial CO_2 tension, and consequent alkalosis. The progressive lowering of the CO_2 dissociation curve expresses the disappearance of alkali from the blood in the attempt of the organism to compensate the alkalosis.

The full significance of these relations can be seen only when they are compared with those in the CO_2 diagrams of acidosis from injections of HCl previously published.[10] In the latter the arterial points deviate to the right of the *OC* line.

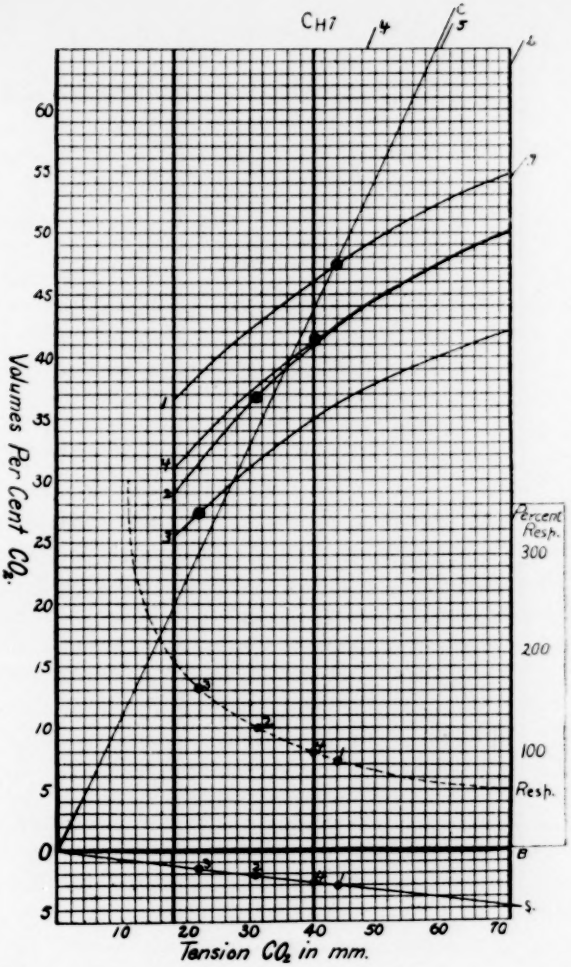

FIG. 2. The CO_2 diagram of the blood under progressively lower tensions of oxygen, Curves 1, 2, and 3; and during recovery when breathing normal air again, Curve 4. From the data of Experiment 2.

Note that under oxygen deficiency the arterial point deviates to the left of the *OC* line, indicating overbreathing and a consequent alkalosis. The compensatory disappearance of alkali from the blood is shown by the progressive lowering of the dissociation curve. Note that on restoration to normal oxygen tension, with the consequent abnormally high ratio oxygen:alkali, the respiration is relatively depressed and the arterial point swings to the right of the *OC* line, thus inducing a condition of acidosis ($H_2CO_3 > NaHCO_3$). Under such conditions it is here seen that alkali is recalled to the blood and the dissociation curve rises.

dying, and required artificial respiration for a few minutes, under which it recovered.

Curve No.	Time.	Oxygen tension.	Blood equilibrated with CO_2 at			Arterial blood.			CO_2 ratio.	C_H[7] (Char. 8).
			18 mm.	40 mm.	72 mm.	CO_2 content.	CO_2 dissolved.	CO_2 combined.		
		mm.	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*vol. per cent*	*per cent*	
1	11.50 a.m.	155	36	49	61	50	2.96	47.04	6.30	0.504
2	2.14 p.m.	92	32	42	53	39	2.02	36.98	5.47	0.437
3	4.10 "	35	27	36	45	27	1.28	25.72	4.97	0.398
4	4.45 "	155	32	47	59	46	2.76	43.24	6.38	0.510

Note that the fall of arterial CO_2 content indicates marked overbreathing which induces a lowered CO_2 ratio and C_H, and is followed by a diminution of blood alkali, as indicated by the CO_2-combining power (Columns 4, 5, and 6). Note also that, after restoration to the normal oxygen tension respiration is depressed below the amount needed for the normal C_H, the CO_2 ratio rises above normal (Column 10), and alkali is recalled to the blood (Columns 4, 5, and 6). The CO_2 diagram which may be plotted from these data is practically identical with Fig. 2.

Overbreathing, Alkalosis, and Hypocapnia.

These experiments afford a striking contrast to those in the first paper of this series.[10] There hydrochloric acid was injected into the blood and the alkali was thus neutralized with a consequent increase of the CO_2 ratio (H_2CO_3 : $NaHCO_3$) and of C_H, and an increase of breathing to remove the excess of CO_2. Here, on the contrary, low oxygen (through the agency of respiratory x which, whatever it is, is evidently not a strong acid) causes first overbreathing and blowing off of CO_2—thus markedly reducing the CO_2 ratio (Column 10) and the C_H. In compensation alkali gradually passes out of the blood and its CO_2-combining power (Columns 4, 5, and 6) gradually falls. In the former experiments with acid the lowering of the blood alkali, hypocapnia, was the first event in the series. Here it is the last.

In Experiments 2 and 3 when a normal oxygen tension was restored the recovery involved the development of a high CO_2 ratio and a supernormal C_H; that is, acidosis. This phenomenon occurred during recovery from injection of hydrochloric acid in the previous series of experiments also,[10] and seems to us to be a point of great theoretical interest in its suggestion as to the process through which the blood alkali and respiration become adjusted to the oxygen tension prevailing at the altitude at which the man or animal lives.

Apparently the stimulant, respiratory x, whatever its character, is not merely the result of reduced oxygen, but rather the resultant of the oxygen and the alkali. Whenever the ratio oxygen: alkali is below normal the amount of respiratory x is increased. Overbreathing occurs, CO_2 is blown off, and the CO_2 ratio falls below normal ($H_2CO_3 < NaHCO_3$). The alkali passes out of the blood until the ratio oxygen : alkali and the ratio H_2CO_3 : $NaHCO_3$ are both normal again. Thus the three principal respiratory factors, pulmonary oxygen tension, alveolar CO_2, and the CO_2-combining power of the blood, are all diminished finally in the same proportion of their sea level values, and equilibrium is thus reattained at a new level. Furthermore the process of seeking equilibrium between these three factors is seen in Experiments 2 and 3 to work equally well for increase of oxygen tension. There is a depression of respiration, a rise of the CO_2 ratio ($H_2CO_3 > NaHCO_3$), a temporary acidosis, and a recall of alkali to the blood.[5,10] Evidently the oxygen tension in the lungs and arterial blood is the fundamental factor controlling the breathing, the alveolar and arterial CO_2 tension, *i.e.* the pulmonary dilution of the CO_2 with air, and the blood alkali. (A more precise statement of the quantitative relations involved in these adjustments will be given in the succeeding paper.)[12]

One or two additional points deserve mention here. We have shown in the first paper of this series[10] that after the blood alkali has been diminished by administration of hydrochloric acid the inhalation of moderate percentages of CO_2 in air—such as do a normal animal no harm whatever—rapidly proves fatal. On the other hand, we have found, both by experiments upon animals

[12] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920, xliii, 29.

and by observations upon men after prolonged and extensive major operations in which the blood alkali has been diminished by overbreathing and subnormal metabolism, that inhalations of CO_2 are markedly beneficial and rapidly recall a normal content of alkali to the blood.[2]

The reactions, beneficial or harmful, to inhalations of CO_2 afford, therefore, an effective method of determining whether a low blood alkali, *i.e.* hypocapnia, has been induced through the acidotic or the acapnial process. Judged by this test recent experiments of Hasselbalch and Lindhard[7] and earlier observations of Mosso[4] indicate that the decrease of blood alkali under low oxygen is of the acapnial and not of the acidotic variety. After exposure to low oxygen for several hours they experienced markedly unpleasant symptoms, *i.e.* mountain sickness, and these effects were distinctly reduced by inhalations of CO_2 in proper dilution. We do not consider, however, that these observations support the claim of Mosso and his followers that CO_2 inhalation during oxygen deficiency is advantageous. In fact we have found that dogs to which CO_2 was administered, even in moderate percentage, during low oxygen experiments, such as those above detailed, were stimulated to violent respiration, and in some cases died suddenly from heart failure.

Apparently the nausea and other discomfort after violent physical exertion are also in part at least due to overbreathing.[13] In experiments with different types of facepiece in gas masks during the war (carried out for the Chemical Warfare Service), we made observations upon a number of university athletes who were accustomed to consistent performance on the running track. The tests consisted in running the half mile and mile against the watch with a pace maker, while the subject was wearing the different types of mask. Without exception the men noticed and voluntarily reported that discomfort after the test was lessened by masks having a moderate dead space. This space was sufficient to decrease somewhat the loss of CO_2 during overbreathing, but not sufficient appreciably to diminish the intake of oxygen.

[13] Collip, J. B., and Backus, P. L., *Am. J. Physiol.*, 1920, li, 568. Campbell, J. M. H., Douglas, C. G., and Hobson, F. G., *Phil. Tr. Roy. Soc. London, Series B*, 1920, ccx, 1.

CONCLUSION.

The process through which oxygen deficiency lowers the blood alkali is as follows: overbreathing blows off an excess of CO_2 (acapnia), leaving the CO_2 ratio, *i.e.* the relation of H_2CO_3 to $NaHCO_3$, and therefore presumably the C_H in the blood, below normal. This alkalosis[14] is then compensated by a disappearance of alkali from the blood.

On restoration to normal oxygen tension the reverse process occurs: the breathing is relatively depressed, the CO_2 ratio (H_2CO_3: $NaHCO_3$) and the C_H of the blood are raised above normal, and this acidosis recalls alkali to the blood.

[14] Haldane, J. S., Kellas, A. M., and Kennaway, E. L., *J. Physiol.*, 1919, liii, 181.

HEMATO-RESPIRATORY FUNCTIONS.

V. RELATION OF OXYGEN TENSION AND BLOOD ALKALI IN ACCLIMATIZATION TO ALTITUDE.

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In the previous paper[1] we have presented evidence which indicates that the tension of oxygen is the fundamental condition to which the organism, slowly but surely, adjusts its respiratory activity, its alveolar tension of CO_2, and the amount of H_2CO_3 in the arterial blood. It thus becomes highly probable also that the tension of oxygen to which a normal man or animal is acclimated determines the amount of blood alkali. Theory demands this, for otherwise the CO_2 ratio (H_2CO_3:$NaHCO_3$) would not be the same in inhabitants of all altitudes, and the C_H of the blood would be different in a man living, for instance, in New York, Denver, or at the summit of Pike's Peak. The investigations of Hasselbalch and Lindhard[2] indicate that with complete acclimatization the C_H of the blood is the same at all altitudes.

In the first paper of this series[3] we attempted to formulate more precisely than hitherto the laws of respiration. The third law, there stated and here to be discussed, is an attempt to define the balance which the various factors in breathing tend to approximate. It is: In physiological equilibrium at all barometric pressures, that is in acclimatization at all altitudes, the tension of CO_2 and amount of alkali in use in the arterial blood vary in direct proportion to the alveolar tension of oxygen, while the volume of true breathing, *i.e.* alveolar ventilation, is inversely proportional.

[1] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1920, xliii, 15.
[2] Hasselbalch, K. A., and Lindhard, J., *Biochem. Z.*, 1915, lxviii, 265, 295.
[3] Haggard, H. W., and Henderson, Y., *J. Biol. Chem.*, 1919, xxxix, 163.

According to this law the general relations under which the C_H of the blood should be constant in a normal individual fully acclimatized, that is in hemato-respiratory equilibrium, at any altitude and barometric pressure may be expressed as follows:

$$(\text{Bar.} - T_{H_2O}) \times F_{RQ} = T_{O_2} = T_{CO_2} \times C_1 = Q_{CO_2} \times C_2 = \frac{1}{100\left(\frac{760 - T_{H_2O}}{\text{Bar.} - T_{H_2O}}\right)} \text{Resp.}$$

In this formula Bar. is the mean atmospheric pressure of the altitude. T_{H_2O} is the tension of water vapor in the lungs at body temperature, 47 mm. at 38°C. F_{RQ} is a factor for the percentage of oxygen in the dry gases of the lungs, with a value usually about 0.14, depending upon the alveolar respiratory quotient. T_{O_2} and T_{CO_2} are the pulmonary tensions of oxygen and CO_2. Q_{CO_2} is the amount of alkali, expressed in volumes per cent of CO_2, called into use in the arterial blood. In the CO_2 diagram it is the ordinate of the point of intersection of the CO_2 dissociation curve and the *OC* line. C_1 and C_2 are constants for the individual, the value of C_1 being about 2.5, and that of C_2, less accurately known and probably subject to wider variations, lying usually between 1.9 and 2.5. 100 Resp. is the volume of true breathing, *i.e.* alveolar ventilation, per 5.5 cc. of CO_2 eliminated at sea level. The denominator of the fraction solves to the relative volume of true breathing at the altitude.

These relations are expressed graphically in Fig. 1.

An extensive series of observations has been made by Fitz-Gerald[4] bearing upon the relations between the mean barometric pressure of any altitude and the alveolar tensions of oxygen and CO_2 of its inhabitants. It covers elevations from sea level up to 11,000 feet; and data from the Pike's Peak expedition[5] extend the series up to 14,000 feet. Her extremely careful technique gives these observations great value. In most of this series, however, no subject, except the investigator herself, could be tested in acclimatization to more than one altitude; and averages

[4] FitzGerald, M. P., *Phil. Tr. Soc. London, Series B*, 1913, cciii, 351; *Proc. Roy. Soc. London, Series B*, 1914–15, lxxxviii, 248.

[5] Douglas, C. G., Haldane, J. S., Henderson, Y., and Schneider, E. C., *Phil. Tr. Roy. Soc. London, Series B*, 1912, ccii, 302.

of inhabitants of Georgia for one group of levels, and of Colorado for another may fairly be expected, considering differences of climate, diet, and other conditions, to show some variations from a purely barometric basis. Even so, all things considered, the diagram which FitzGerald gives to express the relation of alveolar oxygen and CO_2 tensions to the barometer is so near to simple linear proportions that it was from these data that we were led to attempt to formulate the law.

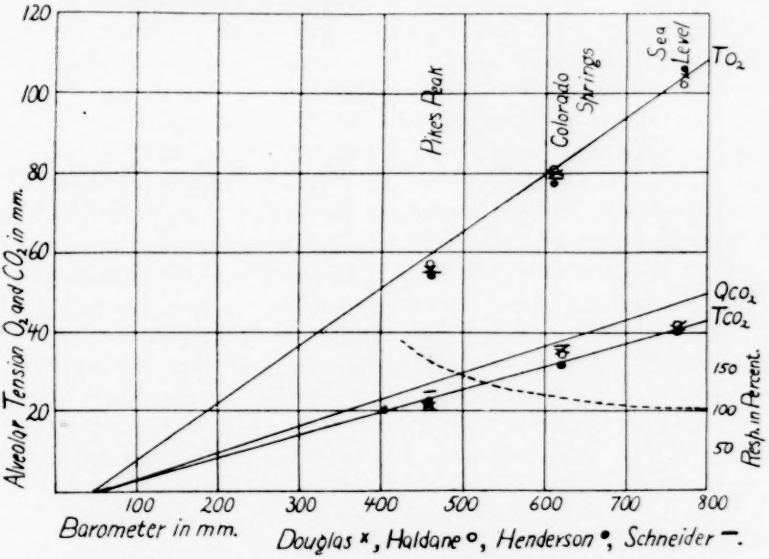

FIG. 1. The relations of the alveolar and arterial oxygen and CO_2 tensions (T_{O_2} and T_{CO_2}), the amount of blood alkali (Q_{CO_2}), and the volume of (true) breathing requisite to full acclimatization to any barometric pressure.

Fortunately, observations upon members of the Pike's Peak expedition[5] afford exactly the data needed. The subjects were approximately acclimated successively at sea level, 14,000, and 6,000 feet. In Fig. 1, the lines T_{O_2} and T_{CO_2} express the requirements of exact proportionality to the barometric pressure minus the water vapor tension of the alveolar air. The points falling on or near these lines show the results of pulmonary air analyses

at sea level and the conditions most nearly approaching acclimatization, that is, after the longest stay for each man, at Colorado Springs and on the summit of Pike's Peak.

It is probable that even in these observations acclimatization was not quite complete, and that the alveolar tensions of CO_2 were therefore a little higher and the tensions of oxygen a little lower than would be the case in complete acclimatization. This is particularly true in Schneider who, as a variation on the conditions of the other members of the expedition, descended twice during the period of investigation. The greater deviation from theory in his case bears out the idea that acclimatization was slightly incomplete in the others also.

At the time the report of the Pike's Peak expedition was written the theory of formation of lactic acid and adjustment of respiration through the acidotic process was apparently the only available explanation of the respiratory readjustments to altitude. Previous papers of this series, in general agreement with the opinion reached independently by Haldane,[6] indicate clearly that the adjustment is through alkalosis and the acapnial process.

The evidence in the literature bearing upon the amount of blood alkali (Q_{CO_2} in Fig. 1) at altitudes is at present less complete and direct than that for the tension of CO_2 but in the main it is clear and decisive. It is contained chiefly in the reports of the Mt. Rosa[7] and Pike's Peak expeditions of 1911 and the more recent work of Hasselbalch and Lindhard.[2] Both of the earlier groups of investigators used the Barcroft method and found the blood alkali to be decreased to such an extent that when a sample of blood was equilibrated with the subject's alveolar air the hemoglobin was approximately mesectic—indicating a normal C_H with a decrease of blood alkali proportional to the decrease of CO_2 tension and inversely proportional therefore to the volume of true breathing.

The relations of hemato-respiratory equilibrium to the barometer, as defined in the law above given and illustrated in Fig. 1, are thus seen to be the limits which the three main factors in

[6] Haldane, J. S., Kellas, A. M., and Kennaway, E. L., *J. Physiol.*, 1919, liii, 181.

[7] Barcroft, J., Camis, M., Mathison, C. G., Roberts, F., and Ryffel, J. H., *Phil. Tr. Roy. Soc. London, Series B*, 1914, ccvi, 49.

respiration, *viz.* the tension of oxygen, the H_2CO_3, and the $NaHCO_3$ in the blood, gradually approach as acclimatization develops. As the experiments in the previous paper show, overbreathing, alkalosis, and the consequent lowering of the blood alkali are the steps through which the changes are effected on passing from a lower to a higher altitude. Slight depression of breathing, high CO_2 ratio (H_2CO_3 : $NaHCO_3$), acidosis, and recall of alkali to the blood are the stages in readjustment on return to sea level.

It must be kept in mind, however, that these readjustments are matters of days or weeks. For the aviator who is at a great altitude for only an hour or two the capacity to develop respiratory *x*, overbreathing, and a low CO_2 ratio seems of the greatest importance.[6,8] The alkalosis tends to shift the affinity of hemoglobin for oxygen and thus assists absorption.

Finally, we may here point out the bearing of these relations upon the nature of that factor in breathing which we call respiratory *x*. We use this term as an alias for the state of the blood, formerly assigned erroneously to the increase of lactic acid, through which alterations of oxygen tension bring about their effects upon the respiratory center. From the relations above shown it appears that the amount of this respiratory stimulant may be subnormal, thus inducing acidosis, as well as supernormal, and that it is dependent not upon the oxygen tension alone, but upon the ratio of the oxygen (T_{O_2}) to the blood alkali (Q_{CO_2}).

If this view is correct it affords an indication as to the line along which we must look for further information regarding this extremely important factor in breathing, in the maintenance of the normal ionic equilibrium of the blood, and in the causation of acidosis when the blood alkali is lowered in disease.[3]

CONCLUSION.

The barometric pressure to which one becomes acclimatized, through the tension of oxygen, is the fundamental factor controlling the volume of air breathed per unit mass of CO_2 eliminated, the alveolar CO_2 tension, and the amount of alkali called into use in the blood. The law of these relations is here formulated, and its bearing upon the unknown anoxemic respiratory stimulant and hyperoxemic respiratory depressant is indicated.

[8] Henderson, Y., *Science*, 1919, xlix, 431.

METHOD FOR DETERMINATION OF CALCIUM IN SMALL QUANTITIES OF BLOOD SERUM.

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In 1916 Marriott, Howland, and Haessler (1) described a micro method for the quantitative estimation of calcium in blood serum. The final determination in their method depends on the property which oxalic acid possesses of decolorizing a solution of ferric thiocyanate. It was not maintained that this method possesses the degree of accuracy expected of a volumetric or gravimetric procedure, but that it is sufficiently accurate to demonstrate such changes in the concentration of calcium in serum as are of clinical significance. In commenting upon their method the authors state (2): "The method is time-consuming and requires a considerable degree of experience in the various manipulations and in the final readings." To remedy these defects the method described below was devised.

Since the work was begun several new methods have appeared in the literature. The procedure described by Halverson and Bergeim (3) requires at least 10 cc. of blood for a single determination and obviously cannot conveniently be used for studies on infants and children. Jansen's (4) method also requires 10 cc. of blood. The introduction of a separate procedure for the preliminary removal of phosphates, as is done in this method and in that of Dienes (5), complicates matters unnecessarily and increases the possibility of error.

The nephelometric method of Lyman (6) has probably been more extensively used in this country for the estimation of calcium in small quantities of blood than any other method. Denis and Minot (7) have reported determinations on the calcium content of the plasma of five normal adult males and one female. They used Lyman's method and found 7.6 to 11.9 mg. of calcium

per 100 cc. of plasma. We have found the concentration of calcium in the serum of normal adults to be singularly constant and hence look upon such striking variations as indicating either that the supposed normal adults were not normal or that the method itself is at fault. Lyman has reported no studies on artificial blood salt solutions, and has made no attempt to recover known quantities of calcium added to serum.

After our method had been completed and in actual use for several months, our attention was called to a method described by de Waard. In his method (8) calcium is precipitated as the oxalate by adding 0.5 cc. of saturated ammonium oxalate, then enough concentrated ammonia until the solution smells ammoniacal on shaking, and finally enough glacial acetic acid until it smells acid. De Waard reports analyses of solutions of blood salts. Each sample contained calcium in amount equal to that found in 4 cc. of serum, or about 8 cc. of blood. No analyses of normal human serum or whole blood are given. No attempt to recover calcium added to blood is reported. It is stated that cow serum contains 8.4 to 8.6 mg. of calcium per 100 cc. of serum. This is in rather striking contrast with a series of determinations reported by Halverson and Bergeim (3). The latter authors never found less than 10 mg. of calcium per 100 cc. of cow serum. It might be pointed out that de Waard's method of precipitation is open to the objections raised by Richards and collabotors (9) and McCrudden (10).

Principle of the New Method.

The blood serum is ashed by the method of Stolte (11) or in the usual manner. The calcium is precipitated by a procedure which in principle is identical with that of McCrudden. A definite amount of 0.1 N oxalic acid in 0.05 N sulfuric acid equal to about four times the equivalent of calcium present is added. The final volume is made up to 2 cc. and an aliquot of the filtrate is titrated with 0.01 N potassium permanganate.

The Method.

1 or 2 cc. of serum or plasma are measured into a platinum crucible, evaporated to dryness on the water bath, and dehydration is completed in the oven at 110°C. The crucible is then

placed in a quartz dish over a Meker or Fisher burner. It is heated gently at first and then more vigorously. The outer dish is then covered with a quartz plate and the heating continued until ashing is complete. The platinum crucible is then removed and the ash dissolved in a small quantity of 1.0 N HCl. It is again evaporated to dryness and ashed as before. A crystalline material is obtained which is readily soluble in not more than 1 cc. of 0.1 N H_2SO_4.[1] The solution is transferred to a Pyrex tube 100 mm. in length and 10 mm. in diameter calibrated for 1 and 2 cc. The tube is heated for a few minutes in the water bath and a drop of 0.01 N potassium permanganate is added to prove the absence of any oxidizable material. The pink color should persist for at least 1 minute. One drop of 0.01 per cent phenolsulfonephthalein is added followed by one drop of concentrated ammonia. The tube is then heated on the water bath to drive off the excess of ammonia. At this point a fluffy precipitate forms. To the tube while hot is added exactly 0.3 cc. of 0.1 N oxalic acid in 0.05 N sulfuric acid. This is added in three portions with shaking after each 0.1 cc. The fluffy precipitate dissolves and is replaced by a copious fine crystalline precipitate. The reaction is usually acid at this point. If it is not, sufficient 0.1 N sulfuric acid is added until the color is lemon-yellow which corresponds to a pH of 6.4 to 6.6. Heating is then continued for a few minutes. The tube is then cooled and 0.1 cc. of a saturated solution of sodium acetate is added and the tube well shaken. The volume is made up to 2.0 cc. and the tube allowed to stand several hours or over night. The material is then filtered through hardened filter paper. 1 cc. of the filtrate is measured into a small beaker. To this, 1 cc. of 20 per cent sulfuric acid (20 cc. of concentrated acid diluted to 100) is added.[2] The beaker is heated on the water bath for a few minutes and then titrated *in good daylight* to a definite pink that persists for

[1] About 0.5 cc. of 0.1 N sulfuric acid is added and solution of the ash facilitated by warming over the steam bath. The solution is then transferred with a small pipette to the Pyrex tube and the process repeated by using two additional portions of 0.25 cc. of 0.01 N sulfuric acid. We have repeatedly demonstrated that transfer of calcium is complete.

[2] Hydrochloric acid should be avoided as it is oxidized to free chlorine by permanganate.

at least 30 seconds. For very accurate work the end-point is determined by comparison with an equal volume of water in a beaker of the same size. The amount of permanganate necessary to give the same intensity of color with an equal volume of water is also determined and this volume is subtracted from the titration. A blank determination should also be made on the reagents. For most purposes the subtraction of 0.04 cc. of 0.01 N permanganate is all that is necessary.

Calculations.

From the permanganate equivalent of 0.3 cc. of 0.1 N oxalic acid is subtracted twice[3] the number of cc. of 0.01 N permanganate used in the final titration, the latter number, however, having been diminished by the amount of permanganate (0.04 cc.) necessary to give a permanent pink color to water. The result is multiplied by 0.2, since 1 cc. of the 0.01 N permanganate is equivalent to 0.64 mg. of calcium oxalate or 0.2 mg. of calcium.

Preparation of Reagents.

0.1 N Oxalic Acid.—0.1 N sodium oxalate solution (Sörenson) is made up and a potassium permanganate solution standardized against it. This solution then serves to standardize the N solution of oxalic acid. This N solution is quite permanent if kept in a cool place in the dark, and from it 0.1 N oxalic acid in 0.5 sulfuric acid may be prepared whenever needed.

0.01 N Potassium Permanganate.—An approximately 0.01 N solution of potassium permanganate is made by diluting a N or 0.1 N solution and the factor determined by titrating against the known 0.1 N oxalic acid solution. Filter before using.

[3] Since half the total quantity is titrated.

PROTOCOLS.

1 cc. samples of Solution B[4] which contained approximately the amount of inorganic phosphate found in blood serum were treated as described in the precipitation method, except that no oxalic acid was added. No precipitate appeared at the end of 48 hours. To three other samples 0.1 cc. of a solution of ammonium phosphate (4.84 gm. to 200 cc.) was added, and the procedure repeated. In spite of this large amount of phosphate no precipitate appeared. Samples of Solution B were analyzed. The results are given in Table I.

TABLE I.

Analyses of 1 Cc. Samples of Solution B (2).

Specimen No.	Calcium.	
	Found.	Present.
	mg.	*mg.*
1	0.190	
2	0.190	
3	0.188	
4	0.188	
5	0.191	
6	0.187	
7	0.190	
8	0.188	
9	0.188	
10	0.190	
Average	0.189	0.193

Known quantities of calcium were added to sera of previously determined calcium content. Results are given in Table II.

[4] Composition of Solution B.

NaCl	4.6340 gm.	KH_2PO_4 was recrystallized and air-dried.
KH_2PO_4	0.0395 "	
$MgSO_4$	0.7577 "	$CaCO_3$ from Iceland Spar.
$CaCO_3$	0.2410 "	All other chemicals "Kahlbaum zur analyse."
HCl concentrated.	10.0 cc.	
H_2O	up to 500 "	

TABLE II.

Recovery of Calcium Added to Serum.

Specimen No.	Calcium.			
	In serum.	Added.	Total present.	Total found.
	mg.	*mg.*	*mg.*	*mg.*
1	0.112	0.04	0.152	0.147
2	0.114	0.04	0.154	0.158
3	0.188	0.193	0.381	0.382
4	0.188	0.193	0.381	0.386
5	0.185	0.193	0.378	0.380

Table III contains a number of determinations on the sera of normal adults and of infants suffering from tetany.

TABLE III.

Calcium Determinations on Normal and Pathological Sera.

Specimen No.	Age.	Diagnosis.	Calcium per 100 cc. serum.
			mg.
1	Adult.	Normal.	9.4
2	"	"	9.6
3	"	"	9.5
4	"	"	9.3
5	"	"	9.9
6	"	"	9.8
7	"	"	9.7
8	Placental blood.	"	11.1
9	" "	"	10.5
10	" "	"	10.4
11	7 yrs.	Acute chorea.	9.4
12	6 mos.	Tetany.	7.2
13	1 yr.	"	3.9
14	6 mos.	"	3.7
15	1 yr.	"	4.2
16	7 mos.	"	6.3
17	10 "	"	5.8
18	10 "	" (treated).	8.1
19	3 yrs.	Epilepsy.	10.7
20	5 "	Multiple neuritis.	9.6

DISCUSSION.

The sources of error in the determination of small quantities of calcium in the presence of magnesium and phosphates are: (*a*) the presence of traces of calcium in the reagents, especially those used in concentrated solution; (*b*) the precipitation of calcium in part as secondary calcium phosphate and of magnesium as magnesium oxalate due to the large excess of oxalate ions and an insufficient degree of acidity; (*c*) calcium oxalate is not entirely insoluble in water. 1 liter of water will dissolve 6 mg. of calcium oxalate (9) and 1 cc. of water containing both ammonium oxalate and ammonium chloride will dissolve at least 0.001 mg. of calcium (5); hence the danger of loss through too energetic washing, especially when a large excess of oxalates is used. (*d*) The preciseness of the end-point in the final titration has been questioned. The exact technique whereby this defect may be overcome has already been described. By using few reagents, errors due to introduction of calcium are avoided. Concentrated sulfuric acid and concentrated ammonia reagents are usually calcium-free, whereas even chemically pure ammonium chloride contains considerable amounts of calcium. Sodium acetate, C. P. (J. T. Baker), can also be obtained calcium-free. The precipitation of magnesium oxalate is avoided by adding 0.1 N oxalic acid in 0.05 sulfuric acid in relatively slight excess in small portions, so that most of it is added after the greater part of the calcium has been precipitated. It is rather surprising that such a slight excess of oxalic acid suffices to precipitate all the calcium. That phosphate is not precipitated under the above described experimental conditions has already been shown. The loss due to washing is, of course, entirely avoided in this method.

CONCLUSIONS.

A method has been described whereby the calcium in 2 cc. of serum may be determined with a maximum error of 3 per cent. In individual determinations of a series the error may often be less than 1 per cent.

Previously reported observations on the calcium content of the blood serum of infants suffering with tetany have been confirmed (2).

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DETERMINATION OF MINUTE AMOUNTS OF ACETONE BY TITRATION.*

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In 1916 a study of the occurrence of acetone bodies in normal blood and urine was undertaken. As a preliminary investigation, a comparison of the different methods of the determination of minute amounts of acetone was made, and certain advantages which a volumetric method possesses over a nephelometric (Marriott, 1913–14 *b*) or a gravimetric one[1] (Van Slyke, 1917) led to the study and extension of the Messinger method given below. Since this modification was first described, various papers have appeared (Ljungdahl (1917), Lenk (1916), Richter-Quittner (1919)) dealing with the same subject.

Many different methods have been proposed for the determination of acetone, and some of these have been adapted to minute quantities of acetone. The method of Scott-Wilson (1911) depends on the formation of a double compound of acetone and mercuric cyanide (Marsh and Fleming-Struthers, 1905), and subsequent titration of the mercury present in the precipitate formed by a modified Volhard technique. This method has been used for the determination of very small amounts of acetone by Marriott (1913–14 *b*), who measured the precipitate with a nephelometer, and by Folin and Denis (1914), who use the colorimeter for the same purpose. In 1898 Denigès proposed a method depending on the formation of a compound of acetone and mer-

* A preliminary report of this method was made before the American Society of Biological Chemists in New York in 1916 (Hubbard, 1917).

[1] When this paper was reported, only a preliminary report of Dr. Van Slyke's method was available.

curic sulfate. The compound was crystalline, and could be weighed, or could be decomposed and the mercury determined by titrating with silver nitrate and potassium cyanide. This method has been studied by Oppenheimer (1899) and by Sammett (1913) and has recently been adapted by Van Slyke (1917) for the determination of minute quantities of acetone, who recommended either gravimetric determination of the final product, or the titration of the mercury by the method of Personne (1863). Engfeldt (1915) proposed a colorimetric method for this determination based on the qualitative method of Frommer (1905), using a color reaction given by acetone and salicylic aldehyde. Csonka (1916) studied this method, but did not find it applicable to very small amounts of acetone. Other reactions are available for demonstrating the presence of small amounts of acetone, and for determining large amounts quantitatively, but are not well adapted for the quantitative determination of very small amounts.

The Messinger (1888) titration method has been most used for the determination of acetone in biological work. This method depends on the formation of iodoform from acetone in an alkaline iodine solution. A known amount of standard iodine solution reacts with acetone in alkaline solution, and the excess of iodine is determined, after acidifying, with a standardized thiosulfate solution. The method based on an older one of Krämer (1880), in which the iodoform was weighed, was proposed by Messinger in 1888, and has since been much studied. The principal objection to it is the number of compounds which give a similar reaction.

Collischonn (1890) believed that the method was inaccurate for small quantities of acetone, but Geelmuyden (1896), Marriott (1913–14 *a*), and others came to the opposite conclusion. Marriott (1913–14 *a*) weighed out small amounts of pure acetone, diluted them with water, and found that values obtained on titration agreed with the theoretical ones. This work was done with solutions of iodine and thiosulfate of approximately 0.1 N concentration.

A few papers have appeared recently in which the use of more dilute solutions is described. Lenk (1916), Ljungdahl (1917), and Richter-Quittner (1919) have mentioned the use of such solutions, but have not described precautions necessary if they are to be used successfully. In a more recent paper Ljungdahl

(1919) has described some of these precautions. He recommends the use of the potassium biiodate solution described by Bang (1913) for the determination of blood sugar, and gives in detail many of the conditions which must be complied with if its use is to be satisfactory. Some of these conditions are the same as those found in working with iodine solutions prepared by the technique described in this paper. A comparison of solutions of biiodate with iodine solutions was made in the course of this work, and led to the conclusion that fewer precautions were necessary when iodine solutions prepared as described were used than when biiodate solutions were used.

0.01 N solutions of iodine cannot be accurately titrated with thiosulfate without the addition of an excess of potassium iodide (Treadwell, 1915), and 0.001 N solutions are even more unsatisfactory. It was found that if a solution of iodine in 3 per cent potassium iodide was used results of titrations were accurate. These solutions were prepared as follows:

A stock solution of iodine in potassium iodide was made by dissolving 13.13 gm. of iodine and 25 gm. of potassium iodide in 1 liter of water, and a solution of sodium thiosulfate of a corresponding strength by dissolving 25.65 gm. of the reagent in 1 liter of water. The solutions are 0.1 N × 103.47 per cent and 1 cc. of either is equivalent to 1 mg. of acetone. They are the solutions described by Shaffer (1908–09).

The thiosulfate solution was kept in a brown bottle connected with a burette by a siphon, and was protected from the air by tubes containing soda-lime. The day after it was prepared it was standardized in the usual way against an equivalent solution of pure potassium biiodate containing 3.362 gm. per liter. The strength of the thiosulfate solution remained unchanged for several months. The iodine solution was standardized against this, and restandardized from time to time, as its strength varied slightly even when kept in a colored bottle.

From these stock solutions dilution was made, using calibrated glassware. Water from a Barnstead still, which has a device for boiling water to expel ammonia and other volatile substances, was used for this dilution, and for all other work with acetone (see also Ljungdahl, 1919). To make the dilute iodine solutions, enough postassium iodide was used to give a final concentration

of about 3 per cent, and the stock solution diluted to $\frac{1}{10}$, $\frac{1}{50}$, or $\frac{1}{100}$ of its original strength. These solutions are equivalent respectively to 0.1, 0.02, and 0.01 mg. of acetone. The thiosulfate solutions of corresponding strength were made by diluting the stock solution with distilled water, and were found to tritrate correctly against the equivalent iodine or biiodate solutions. The strength of the iodine solutions remained unchanged for 2 or 3 days; after that it often increased slightly, probably due to oxidation of the relatively large amounts of potassium iodide present. The thiosulfate solutions were permanent for from 24 to 48 hours, but not for a longer time. No special precautions

TABLE I.

Effect of Strength of Acid and of Time on the Titration of Dilute Iodine Solutions.

Iodine.		Thiosulfate.	Acid concentration (H_2SO_4).	Titrated at once.	Titrated after 30 min.
Strength.	Amount.	Strength.			
	cc.			cc.	cc.
0.001 N	10	0.001 N	0.5 N	10.0	10.8
0.001 N	10	0.001 N	0.25 N	9.93	9.97
0.001 N	10	0.001 N	Slight excess.	9.97	9.93

Volume of each solution equals 50 cc.

were taken in preparing these solutions, as it was found more convenient to make a freshly diluted solution of thiosulfate daily.

In titrating with these dilute solutions certain precautions were necessary. All solutions of sodium hydroxide gave a slight blank, the size of which depended on the amount and grade of the sodium hydroxide used. It was found that this blank was constant if the alkaline iodine solutions were allowed to stand for 10 minutes, and that it did not increase in $\frac{1}{2}$ hour; if a good grade of sulfuric acid in not too great excess (a final excess of 0.25 N or less) was used for acidifying; and if the solution was titrated within 15 minutes after adding the acid.[2] Table I shows the effect of excess of acid on the titration of dilute iodine solutions.

[2] Some grades of sulfuric acid contain substances which react with iodine solutions, and it was found advisable to allow the acidified solution to stand for 5 minutes before titrating. If the sulfuric acid is boiled for 10 minutes, this error is decreased.

TABLE II.

Effect of Volume and Time on the Titration of Alkaline Iodine Solutions.

Solutions.			50 cc. volume.			100 cc. volume.			150 cc. volume.			200 cc. volume.		
Iodine.		Thiosulfate.	½ min.	5 min.	30 min.	½ min.	5 min.	30 min.	½ min.	5 min.	30 min.	½ min	5 min.	30 min.
Strength.	Amount.	Strength.												
	cc.		cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
0.01N	10	0.01N	9.97		9.95	9.93		9.92	9.92		9.88	9.92		
0.01N	20	0.01N	19.83		19.87	19.87			19.92		19.88	19.90		19.72
0.002N	5	0.001N	9.80	9.15	9.10									
0.002N	10	0.001N	19.55	19.00	18.92	19.65	19.00	18.80	19.67	18.80				

1 cc. of a strong sodium hydroxide solution (100 gm. of NaOH+100 cc. of H_2O) added, and the alkaline iodine solution allowed to stand as shown before acidifying.

The volume of the solution also affects the value of the blank in certain cases. If 0.001 N iodine solutions are used, the volume of the solution must be kept practically constant; when 0.002 N solutions are used, the volume may vary from 50 to 100 cc., but should not be more dilute; with 0.01 N solutions the variation may be very much greater. The value of the blank is so small that the determination with the stock solutions is not affected (Table II).

When small amounts of acetone were determined, it was found necessary to insure the addition of sufficient alkali. Table III gives some results obtained with a solution of acetone containing

TABLE III.

Effect of the Concentration of Alkali and of Time on the Formation of Iodoform.

Stood alkaline.	Acetone present.	Approximate normality.					
		0.2 N NaOH found.	0.15 N NaOH found.	0.12 N NaOH found.	0.10 N NaOH found.	0.07 N NaOH found.	0.04 N NaOH found.
min.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
5	0.450	0.450	0.395		0.443		
7	0.450					0.415	
10	0.450	0.438	0.452 0.439 0.445	0.448	0.447	0.439	0.382
15	0.450	0.449			0.455		
20	0.450	0.448	0.452		0.435	0.448	
30	0.450		0.455				

10 cc. of 0.01 N iodine used in this determination in each experiment.

about 0.5 mg. A final concentration of 0.1 N to 0.2 N sodium hydroxide gave complete formation of iodoform in 10 minutes, while smaller concentrations did not give complete formation within that time. The limitations described above and illustrated in Tables I to III were the only limitations found to the application of the method.

Method.

To acetone, contained in a volume of 50 to 100 cc., a known amount of iodine was added. If there was probably very little acetone present—such amounts as are obtained from a few cc.

of normal blood or urine—10 to 25 cc. of 0.00207 N (1 cc. = 0.02 mg. of acetone) iodine, diluted as described, were used; for amounts of acetone from 0.2 to 2.0 mg., such as are obtained from blood or breath of patients with a marked acetonemia, 25 cc. of a solution five times as strong were added; for larger amounts, such as are found in the urine of patients with marked acetonemia, the stock solutions as described by Shaffer (1908–09) were used. When amounts of acetone larger than 0.2 mg. were present it was found that the volume of solution in which the acetone was contained had little effect on the determination, and if several milligrams were present the reaction could be carried out in 300 cc., as accurately as in 50 cc. No matter what amount of acetone is present, enough iodine must be used to give a distinct excess of the reagent.

To the solution containing acetone and iodine, 2 cc. of a sodium hydroxide solution, made by dissolving 200 gm. of sodium hydroxide in 300 cc. of water, were added, and the solution was shaken well for a few seconds. Long shaking was not found necessary. After the solution had stood alkaline for 10 minutes or more, 1 to 2 cc. of sulfuric acid (1 part of concentrated acid to 1 part of water) were added, and the solution was titrated with 0.001, 0.01, or 0.1 N sodium thiosulfate. A little clear dilute starch solution was added before the end of the titration to serve as indicator. A blank was run every day to test the relative strength of the iodine and thiosulfate solutions, and the result calculated from the difference in value between the titration of this blank and that of the solution containing acetone. As stated above, dilute solutions of sodium thiosulfate were prepared daily.

Acetone solutions used were carefully purified by redistilling repeatedly from fused calcium chloride until the product boiled sharply at 56°C. Samples of this product, when weighed out in small pipettes, sealed, the seal broken under water, and the acetone solution made to volume after the method of Marriott (1913–14 *a*), gave values on titration that agreed with the theoretical ones. The samples analyzed were of the magnitude of 20 mg. Solutions of this pure acetone were diluted to appropriate strength, and were used for the determination of minute amounts of acetone. It was necessary to restandardize the strong solutions frequently, as all acetone solutions lose strength readily.

Tables IV and V give results obtained by the method described. The figures in the column headed "Present" correspond with figures calculated from parallel determinations carried out on the strong acetone solutions from which the dilute solutions used were prepared.

Since it was desired to test the effect of repeated distillation from various solutions upon acetone, a few experiments were carried out on the loss of acetone when exposed to the air under

TABLE IV.

Results on Solutions of Pure Acetone.

Solutions used.	Present.	Found.
	mg.	*mg.*
0.00207 N iodine and 0.001035 N thiosulfate.	0.0046	0.0049
	0.00916	0.0104
	0.0229	0.0227
	0.0458	0.0458
	0.0916	0.0900
	0.2290	0.2345
0.01035 N iodine and 0.01035 N thiosulfate.	0.045	0.038
	0.090	0.082
	0.225	0.218
	0.450	0.450
	0.900	0.887
	2.250	2.235

various conditions. It appears from the table that the percentage loss of acetone under constant conditions varies with the volume of the solution used, but is practically independent of the amount of acetone present. The loss varies with the amount of surface of the solution and with temperature, as was expected, but, if the volume of solution is 100 cc., there is very little more loss at 37°C. than at 20°C. (see Table V). If solutions were sealed there was no change in acetone content of jars exposed to room temperature for 2 hours.

TABLE V.

Loss of Acetone on Standing.

Volume.	Acetone.	Time of standing. ½ hr.		1 hr.		2 hrs.		Notes.
cc.	*mg.*	*mg.*	*per cent*	*mg.*	*per cent*	*mg.*	*per cent*	
50	7.15	6.79	95	6.49	91	5.69	80	In 200 cc. Erlenmeyer flasks.
50	0.674	0.621	92	0.601	89	0.546	81	
100	7.15	7.06	99	6.95	97	6.58	92	
100	0.674	0.663	99	0.653	97	0.628	93	
50	6.43	5.82	91	5.47	85	4.87	76	In small specimen jars, diameter 6 cm.
100	6.43	6.12	95	5.92	92	5.52	86	
150	6.43	6.27	98	6.04	94	5.72	89	
50	6.43	3.85	60	3.30	51	1.75	27	In large specimen jars, diameter 16 cm.
100	6.43	4.70	73	4.40	68	2.85	44	
150	6.43	4.98	77	4.85	75	3.50	55	
50	8.15					6.20	76	Small specimen jars at room temperature, 20°C.
50	20.60					15.75	76	
100	8.15	7.65	94	7.20	88	6.70	82	
100	20.60	19.42	94	18.60	90	17.17	83	
50	8.15			6.10	75			Small specimen jars heated to 38°C. in incubator.
50	20.60			14.95	73			
100	8.15			7.20	88			
100	20.60			17.98	87			

Distillation of Acetone from Various Oxidizing Reagents.

In order to separate acetone from various other compounds which also react with alkaline iodine solutions, it may conveniently be distilled from various reagents. Table VI gives results obtained by distilling acetone solutions from sodium peroxide; sulfuric acid plus potassium permanganate; sulfuric acid plus potassium dichromate; and by repeated distillation from sodium peroxide. The amount of acetone under the heading "Control" was measured and titrated simultaneously with the specimen oxidized. Table VI shows that acetone is not oxidized, under the conditions described, by sodium peroxide or by sulfuric acid plus potassium dichromate, or by sulfuric acid plus potassium

permanganate if the concentrations of acid and permanganate are properly regulated; if the volume is about 150 cc. and the concentration of sulfuric acid about 0.5 N, and 0.2 gm. of potassium permanganate is added, from 0.07 to 20 mg. of ace-

TABLE VI.

Distillation of Acetone from Oxidizing Reagents.

From Na_2O_2.*			From $H_2SO_4 + KMnO_4$.					Notes.
Na_2O_2	Control.	Found.	Volume.	H_2SO_4	$KMnO_4$	Control.	Found.	
gm.	*mg.*	*mg.*	*cc.*	*cc.*	*gm.*	*mg.*	*mg*	
			150	25	Few crystals.	7.68	7.70	
0.25	6.90	6.70	150	25	0.1	7.70	7.40	
0.25	6.78	6.80	150	25	0.2	7.70	6.90	
0.25	6.70	6.75	150	10	0.1	19.82	19.60	H_2SO_4 is about 20×N. Made by diluting 1 part H_2SO_4 with 1 part water.
0.5	0.0757	0.0749	150	10	0.2	19.87	19.17	
0.5	3.64	3.70	150	5	0.2	0.0757	0.0763	
0.5	6.85	6.85	150	5	0.2	0.682	0.682	
0.5	17.38	17.25	150	5	0.2	19.82	19.60	
0.5	18.60	18.32	150	5	0.3	7.05	6.92	
0.5	35.80	36.15	100	10	0.1	7.85	7.65	
1.0	6.73	6.55	100	5	0.2	20.02	19.54	
1.0	6.78	6.78						
1.0	14.05	14.10						
2.0	7.10	6.25						
2.0	6.83	6.97						

From $H_2SO_4 + K_2Cr_2O_7$.				
Volume.	H_2SO_4	$K_2Cr_2O_7$	Control.	Found.
cc.	*cc.*	*gm.*	*mg.*	*mg.*
150	25	1	0.816	0.827
150	25	1	7.60	7.67
150	25	1	19.95	19.80
100	25	1	7.60	7.53

	Na_2O_2	Control.	Found.
	gm.	*mg.*	*mg*
Distilled from Na_2O_2 and redistilled three times from the same amount of the reagent.	0.5	0.605	0.610
	0.5	5.60	5.20
	1.0	6.88	6.85
	1.0	6.72	7.07

* Volume of 100 cc.

tone may be recovered quantatively. If much greater concentrations of acid and permanganate are used, there is some loss of acetone, but the method can be applied safely to the separation of acetone from substances which are more easily oxidized by sulfuric acid-permanganate solution than acetone is.

Table VII shows more in detail the effect of prolonged exposure to some of these oxidizing reagents. A known amount of acetone was added to each of several flasks, with water to the volume indicated. Oxidizing reagents were then added, the flask was connected with a condenser and receiver, and placed on a boiling water bath. After the solution had stood in boiling water for from 10 to 30 minutes, the water bath was removed without disconnecting the flask, and the contents of the flask were distilled actively for 10 minutes. The acetone in the receiving flask was then titrated by the method described. Not more than 10 per cent of the acetone used was found in the receiving flask when an alkaline, acid, or neutral solution was heated on a boiling water bath for 30 minutes connected in this way. Controls were run as in the preceding experiments.

Table VII shows that sodium peroxide, except in very large amounts, does not oxidize acetone, nor do the concentrations of sulfuric acid and potassium dichromate tested. The table brings out more clearly than does the preceding one the effect of concentration of sulfuric acid and potassium permanganate in independently increasing oxidation. The concentrations of these two substances which gave no oxidation on distillation gave oxidation when heated under these conditions.

Table VIII presents the effect of the following treatment. Acetone was distilled from acid and redistilled from alkali to a volume of about 150 cc. To this distillate were added 5 cc. of sulfuric acid diluted with 1 part of water and 0.2 gm. of potassium permanganate; this was distilled to give a volume of about 100 cc. in the distillate. To this distillate was added 0.5 gm. of sodium peroxide and this was again distilled. This distillate was titrated by the method described.

The following substances treated as described above gave no reaction with alkaline iodine solutions: 10 cc. of saturated chloroform, gasoline, benzene, toluene solutions; 500 mg. of phenol, methyl alcohol, formaldehyde; about 400 mg. of ethyl alcohol

TABLE VII.

Oxidation of Acetone on Boiling Water Bath.

Sodium peroxide.

Heated 10 min. on water bath.					Heated 30 min. on water bath.				
Acetone.		Volume.	Na_2O_2	Found.	Acetone.		Volume.	Na_2O_2	Found.
Initial.	Controls.				Initial.	Controls.			
mg.	*mg.*	*cc.*	*gm.*	*mg.*	*mg.*	*mg.*	*cc.*	*gm.*	*mg.*
		100	0	6.55			100	0	6.80
		100	0.25	6.62	7.20	6.68	100	0.25	6.68
7.12	6.54	100	0.50	6.82		6.80	100	0.50	6.80
	6.70	100	1.00	6.42			100	1.00	6.75
		100	2.00	5.94					
		75	0.50	6.62	7.12	6.60	100	2.00	6.05
		150	0.50	6.64		6.40			

Potassium permanganate and sulfuric acid.*

Acetone.		Volume.	Time of heating.	Acid.	$KMnO_4$	Found.
Initial.	Controls.					
mg.	*mg.*	*cc.*	*min.*	*cc.*	*gm.*	*mg.*
		150	10	0	0.1	6.59
		150	10	5	0.1	6.27
		150	10	5	0.2	5.92
7.17	6.57	150	10	10	0.1	6.19
	6.57	150	10	10	0.2	5.49
		150	10	10	Few crystals.	6.97
		150	10	25	" "	7.07
		150	10	25	0.1	5.37
7.10	6.91	150	30	5	0.1	6.08

Potassium dichromate and sulfuric acid.*

Acetone.		Volume.	Time of heating.	Acid.	$K_2Cr_2O_7$	Found.
Initial.	Controls.					
mg.	*mg.*	*cc.*	*min.*	*cc.*	*gm.*	*mg.*
7.87	7.57	150	30	25	1	7.55
	7.62	150	30	25	1	7.75

* Sulfuric acid is about 20×N.

(probably partially removed by the repeated fractional distillation (Richter-Quittner, 1919) and partially by oxidation), 50 mg. of acetaldehyde, and 50 mg. of ether.[3] Various normal urines and bloods have been analyzed by this technique, and they gave values of from 0.01 to 0.03 mg. of acetone in the 10 cc. samples taken.[4]

TABLE VIII.

Successive Distillation of Acetone from Different Reagents.

Reagents used.	Control.	Found.
	mg.	*mg.*
Distilled successively from H_2SO_4, NaOH, H_2SO_4+$KMnO_4$, and Na_2O_2.	0.264	0.248
	0.664	0.669
Control measured and titrated when the solution was. It was exposed to the air during oxidation and distillation of the solution studied.	0.664	0.661
	1.303	1.361
	1.605	1.608
	3.44	3.44
	18.05	17.95

CONCLUSION.

A method has been described by which the Messinger titration method may be applied to dilute acetone solutions, certain reactions of acetone have been studied, and a method has been proposed by which small amounts of acetone can be separated from much larger amounts of alcohol and other interfering compounds.

My thanks are due to Dr. Philip A. Shaffer for his advice and assistance in this work.

[3] Ether is oxidized by acid permanganate solutions as it is by dichromate solutions to give a compound which reacts like acetone (Short, 1920). If the ether present is less than 50 mg., this compound is removed by the subsequent treatment with sodium peroxide.

[4] A paper in which this technique is applied to normal urine and blood is in preparation.

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DETERMINATION OF ACETONE IN EXPIRED AIR.*

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The fact that acetone is present in large amounts in the breath of patients suffering from severe diabetes has been known for a long time, and the high percentage of acetone which might occur in the breath was demonstrated by Geelmuyden in 1897. He injected acetone into rabbits, and showed that as much as 75 per cent of the acetone recovered might be found in the breath. In spite of these facts there are comparatively few quantitative determinations of acetone recorded in the literature, and almost all of these are in severe cases of diabetes.

One of the reasons for this is a lack of a convenient method for determining such small amounts of acetone as are found in the breath of normal subjects, or of subjects suffering from a moderrate degree of acetonemia. Nebelthau (1897) had patients suffering from diabetes breathe directly through alkaline iodine solutions, after which he determined the amount of acetone by the Messinger (1888) titration. He admitted that this method was inexact, due to loss of iodine. Such a method can, however, be used to show the presence of acetone in breath, and has been adapted for its determination when present in large amounts by Marriott (1914), and for the determination of acetone in the air in chemical plants by Elliott and Dalton (1919). Scott-Wilson (1911) used the mercuric cyanide reagent described by him to demonstrate the presence of acetone in the breath of patients suffering from "acidosis," but did not describe a technique for a quantitative method. Geelmuyden (1897) described a method

* A preliminary report of this work was given before the meeting of the American Society of Biological Chemists in Baltimore in April, 1919.

Part of the work was carried out in the chemical laboratory of Harvard University through the courtesy of Dr. L. J. Henderson in February, 1919.

in which air from the lungs was oxidized in a combustion furnace, and the acetone calculated from the carbon dioxide so produced. He experimented with small animals in a Pettenkofer-Voit calorimeter. The air, drawn from the chamber through sodium hydroxide, passed to the furnace, where it was oxidized, and the carbon dioxide so obtained was caught in a second series of bottles of sodium hydroxide. Müller (1897–98) developed a technique which might be used with human subjects. He caused the patient to breath through a series of four Woulff bottles containing 250 to 500 cc. of water kept cold by ice. The pressure of the water in the bottles was reduced by a pump capable of moving a large volume of air, and subjects could breathe for ½ to 1 hour without discomfort. Acetone in the bottles was determined by the Messinger titration. This technique included in the determination other substances which react with alkaline iodine solutions and which may be present in the breath. Voit (1899) used this method of Müller, in connection with the Pettenkofer-Voit respiration chamber, to determine the effect of diet upon the excretion of acetone by small animals.

Folin and Denis (1915), in a study of the metabolism of two fasting women, have used a method based on the retention of acetone by a sodium bisulfite solution, and its subsequent determination with Scott-Wilson reagent, as described by them (Folin and Denis, 1914). Their patients breathed for 3 minutes through 10 cc. of 0.5 per cent sodium bisulfite solution. The results showed a marked increase of acetone during fasting, but did not show acetone in the breath of these patients on a normal diet. The method described below resembles this one in many essential features.

Folin and Denis (1914) have stated that acetone is retained almost as completely when blown through a 2 per cent solution of sodium bisulfite as is ammonia by a solution of hydrochloric acid. Preliminary tests showed that acetone in a stream of carbon dioxide from a Kipp generator was quantitatively retained by such a solution in spite of the decomposition of bisulfite.

Experiments were also made on the recovery of acetone added to sodium bisulfite solutions after distillation from sulfuric acid plus potassium permanganate and from sodium peroxide successively, a procedure which separates acetone from rather large

amounts of alcohol, acetaldehyde, and other interfering compounds.[1] To known amounts of acetone plus 2 gm. of sodium bisulfite in about 150 cc. of water, 10 cc. of 10 per cent sodium hydroxide were added, and the solution was distilled. The distillate was redistilled successively from sulfuric acid plus potassium permanganate and from sodium peroxide. Table I shows the results of such experiments. The maximum loss of acetone was about 6 per cent, and was not due to oxidation, as the same loss was shown by the control solutions exposed to the air.

TABLE I.

Recovery of Acetone Added to Bisulfite Solutions.

Description.	Acetone added.	Acetone control	Thiosulfate.		Acetone recovered.
	mg.	*mg.*		*cc.*	*mg.*
2 gm. of $NaHSO_3$+acetone as shown,	0.0			0.57	0.011
distilled from NaOH, and redistilled successively from H_2SO_4+	0.0			0.88	0.018
$KMnO_4$, and from Na_2O_2 as described. Control column gives	0.0		0.002N*	1.10	0.022
results from acetone in about 100 cc. of water exposed to the air	0.0			0.45	0.009
during the experiment. First six	0.0			0.70	0.014
results show blanks by this	0.0			0.56	0.011
method.	0.096	0.087	0.002N*	5.01†	0.102
	0.685	0.670	0.01N*	6.67†	0.667
	7.40	7.02	0.1N*	6.92†	6.92
	7.40	6.82	0.1N*	7.12†	7.12
		17.50	0.1N*	17.18†	17.18

* Approximate normality, true figure 103.47 per cent of these.

† Corrected for the blank given by the reagents.

Method.

For determining acetone in air, 75 cc. of a freshly prepared 2.5 per cent sodium bisulfite solution were measured into each of two bottles. The patient breathed through a mask (a celluloid etherizing mask with the valve removed), or through a mouthpiece. He received air through a simple valve placed in a vertical position to insure complete closure with expiration. The glass tubing used was 9.5 mm. and the bottles were 75 mm. in diameter. The amount of glass and rubber tubing was reduced to a minimum.

[1] Hubbard, R. S., *J. Biol. Chem.*, 1920, xliii, 43.

After the subject had breathed for 5 or 10 minutes (usually for 10 minutes) the bottles were disconnected, 10 cc. of 10 per cent sodium hydroxide were added to each, and the contents of each were washed separately into a 500 cc. Kjeldahl flask and treated as described below to remove interfering compounds already referred to. The solution was first distilled for 10 minutes through a water-cooled condenser into a second Kjeldahl flask containing enough water to cover the end of the receiving tube (Marriott, 1913–14 *a*) to give a final volume of about 150 cc. To the contents of the receiving flask 5 cc. of sulfuric acid (one part of concentrated acid plus one part of water) and about 0.2 gm. of potassium permanganate were added. The solution was then redistilled with the same precautions to give a volume of about 100 cc. in a third distilling flask. To this flask 0.5 gm. of sodium peroxide was added, and a final distillation made. This final distillation was made into a little distilled water to give a volume of between 50 and 100 cc., and the acetone determined by the technique described in the preceding paper,[1] or it was made into 25 cc. of Scott-Wilson reagent,[2] and the acetone estimated by the degree of turbidity produced.

If the latter method was used, standards containing approximately the same amount of acetone were distilled into the same amount of reagent; all solutions were made to 100 cc., and the turbidities produced were compared in Nessler tubes. By this method readings could be made within 0.005 mg. For more exact determinations, solutions were compared with the nearest standard either in a nephelometer (Marriott, 1913–14 *b*), or, if more than 0.2 mg. of acetone was present, in a colorimeter according to the technique of Folin and Denis (1914).

Usually slight blanks were obtained whether the acetone was determined by the Messinger titration or by the turbidity method. Blanks by the iodine method are given in Table I, blanks by the turbidity method were usually about 0.007 mg., and were prob-

[2] Scott-Wilson's reagent may be prepared as follows (Marriott, 1913–14 *a*, p. 284, foot-note): "Mercuric cyanide, 10 grams; Sodium hydroxide, 180 grams; Water, 1200 cc. The solution is agitated in a flask and 400 cc. of a 0.7268 per cent solution of silver nitrate slowly run in." The solution should be prepared at least 24 hours before it is used, and should be filtered if it is not perfectly clear.

ably produced by a trace of ammonia. The source of the blanks found with the iodine method was in the distillation from sodium peroxide, and probably arose from oxidation of some compound (possibly fat) which produced a substance that reacted with alkaline iodine solutions. To keep this blank constant, the flasks used for the last distillation must be kept free from grease, and the amount and approximate concentration of sodium peroxide must be kept fairly constant. Table II gives results on a series of normals in which the two methods are compared. The values are corrected for the blanks.

TABLE II.

Comparison of Results by Iodine and Turbidity Methods.

Subject.	Turbidity method. Found.	Iodine method. Found.
	mg.	*mg.*
R. S. H.	0.035	0.040
M. L.	0.015	0.015
Mrs. S.	0.007	0.007
Dr. O.	0.025	0.025
N. B.	0.025	0.025

Each subject breathed for 10 min. in both tests.

To find out whether the oxidation by permanganate and sodium peroxide caused any change in the value obtained, samples of breath collected as described were distilled from alkali and acid only, to remove sulfurous acid and ammonia, and other samples of breath, obtained as nearly simultaneously as possible, were analyzed by oxidation. By iodine titration values as much as 50 per cent lower were obtained after oxidation; by the turbidity method, results after oxidation agreed with those obtained after distillation within 0.005 mg. There is, therefore, in normal breath a substance, not acetone, which reacts with alkaline iodine solutions, and which is removed by oxidation with acid permanganate and sodium peroxide. This substance does not react with Scott-Wilson's reagent. The agreement of the results obtained by Scott-Wilson's reagent after oxidation with results after distillation shows an absence in the breaths studied of appreciable amounts of secondary propyl alcohol, or other compounds

which can give acetone on oxidation with potassium permanganate.

To show the completeness with which acetone can be recovered from the breath by the apparatus described, two experiments were run. First, a normal subject breathed through three bottles of 2.5 per cent sodium bisulfite instead of two, and the contents of the third bottle were analyzed with negative results; second, a bottle containing a known amount of acetone in 75 cc. of water was placed between the valve and the first bottle containing bisulfite. Table III gives a record of several such experiments, and shows a high percentage of recovery of relatively large amounts of acetone.

TABLE III.

Recovery of Acetone Added to Breath.

Description.	Present.	Residue.	1st bottle.	2nd bottle.	Total recovered.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Breathed 10 min. through bottle containing known amount of acetone into two bottles containing $NaHSO_3$ solution.	3.88	1.43	0.98	0.83	3.24
	3.88	1.66	1.40	0.41	3.47
	2.65*	0.75	1.25	0.70	2.70
	2.65*	0.30	1.70	0.50	2.50
	2.65*	0.30	1.65	0.45	2.40
	1.50	0.78	0.51	0.28	1.57

* Experiments in which acetone was warmed to 50° C.

To prove the efficiency of the mask, a bottle containing 75 cc. of 2.5 per cent sodium bisulfite was connected with the Benedict portable respiration apparatus (a closed circuit respiration system) (Benedict, 1918) in such a way that the air from the subject passed through this bottle before it passed through the bottle of soda-lime used to absorb CO_2. By this arrangement sulfur dioxide was absorbed, and did not reach the subject's mouth. Preliminary tests showed that as much as 5 mg. of acetone introduced into the machine were recovered quantitatively. Comparative tests using this calorimeter, and the mask and two bottles, gave the same value for acetone within 0.002 mg. Experiments were run to determine whether the presence of this bottle of

sodium bisulfite had any effect on the determination of metabolism by the apparatus, and showed that there was no such effect.

Table IV shows the results of the method on a series of normal subjects. These results were obtained by using the face mask, and analyzing with Scott-Wilson's reagent after oxidation with

TABLE IV.

Results of Turbidity Method on Normal Subjects.

No. and sex.	1st bottle.	2nd bottle.	Total determination.	Per hour.
	mg.	*mg.*	*mg.*	*mg.*
4 ♂	0.068	0.013	0.081	0.49
5 ♀	0.018	0.011	0.029	0.17
6 ♀	0.018	0.008	0.026	0.16
9 ♂	0.042	0.013	0.055	0.33
10 ♂	0.033	0.010	0.043	0.26
11 ♀	0.033	0.010	0.043	0.26
13 ♂	0.015	0.008	0.023	0.14
14 ♀	0.038	0.010	0.048	0.29
15 ♀	0.038	0.010	0.048	0.29
16 ♂	0.023	0.008	0.031	0.19
18 ♀	0.028	0.008	0.036	0.22
21 ♂	0.023	0.010	0.033	0.20
22 ♂	0.038	0.018	0.056	0.34
23 ♀	0.023	0.005	0.028	0.17
24 ♀	0.023	0.010	0.033	0.20
25 ♂	0.028	0.008	0.036	0.22
28 ♀	0.028	0.011	0.039	0.23
35 ♀	0.028	0.005	0.033	0.20
37 ♂	0.023	0.003	0.026	0.16
38 ♀	0.028	0.013	0.041	0.25
40 ♂	0.068	0.018	0.086	0.52
42 ♀	0.053	0.028	0.081	0.49

Each subject breathed 10 min.

potassium permanganate and sodium peroxide. The results are corrected for the slight blank given by the reagents.

Table V shows the agreement which was found between duplicates, when the subject breathed for different periods within the same hour, and the different results obtained on the same subject on different days. A few results on cases showing an acidosis are included.

TABLE V.

Acetone in Breath.

No.	Subject.	Date.	Breathed.	1st bottle.	2nd bottle.	Total.	Per hour.	Remarks.
		1919	min.	mg.	mg.	mg.	mg.	
3	A ♂	Apr. 15	10	0.028	0.013	0.041	0.25	Normal subject.
33		" 19	10	0.063	0.018	0.081	0.49	Nos. 33 and 34 determined in the same hour.
34		" 19	5	0.038	0.011	0.049	0.59	
39		" 20	10	0.038	0.023	0.067	0.37	
1	B ♂	" 12	10	0.03	0.01	0.040	0.24	Normal subject.
2		" 12	10	0.025	0.005	0.030	0.18	
29		" 16	2	0.023	0.005	0.028	0.84	Nos. 29, 30, and 31 done within the same hour.
30		" 16	10	0.112	0.028	0.140	0.84	
31		" 16	5	0.058	0.018	0.076	0.91	
32		" 19	10	0.063	0.023	0.086	0.52	
17	C ♀	" 16	5	0.193	0.020	0.213	2.6	Diabetic; Legal positive.*
19	D ♀	" 16	5	0.010	0.003	0.013	0.16	" " negative.*
20	E ♀	" 16	5	0.058	0.023	0.081	0.97	" " positive.*
43	F ♂	" 21	5	1.113	0.133	1.25	15.0	Exophthalmic goiter.
44	G ♂	" 22	10	0.713	0.164	0.88	5.24	Diabetic; Legal positive.*

* Legal's reaction done on urine at the same time that the breath sample was obtained.

SUMMARY.

In the paper given above a convenient method is described by which acetone can be determined in the expired air by iodine titration or by precipitation with Scott-Wilson's reagent after removal of such interfering compounds as primary alcohols, aldehydes, phenols, and the simple cyclic hydrocarbons. Close agreement of results obtained by these chemically different methods of determination on the breath of normal subjects indicates strongly that the compound so determined is acetone. A series of results obtained by the method on normal subjects and on a few pathological cases is included.

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BIOCHEMICAL CHANGES IN THE FLESH OF BEEF ANIMALS DURING UNDERFEEDING.*

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INTRODUCTION.

There have recently appeared in the literature two papers dealing with the biochemical changes in the muscle of the salmon during the fast of spawning migration. The first paper[1] showed that besides experiencing a loss of muscular tissue these fish exhibited an impoverishing of the muscular tissue that remained to the extent of from 10 to 30 per cent of the protein content. The organic extractives nevertheless remained rather constant in percentage of the tissue and even increased under certain conditions. The second paper[2] confirmed the work presented in the former and showed further that the concentration of amino nitrogen relative to the water content after an initial increase remained constant throughout the migration. The first investigator argued that the results indicate a storage of reserve protein in the salmon muscle.

The Material.

The data presented in this paper were derived from material collected and analyzed during the course of an extended experiment on the uses to which a beef animal puts its food. A part

* Published with the permission of the Director of the Agricultural Experiment Station.

Presented before the Division of Biological Chemistry, American Chemical Society, St. Louis, April 14, 1920.

[1] Greene, C. W., *J. Biol. Chem.*, 1919, xxxix, 435.

[2] Greene, C. H., *J. Biol. Chem.*, 1919, xxxix, 457.

of these data has been published in another connection.[3] To this are added data concerning the classes of protein and the glycogen content in the muscles of the animals.

All the animals were of the Hereford-Shorthorn type of beef steer. Six of them had been very fat at 11 months of age. The one which was least fat was killed as a control (No. 1) while the others were used in a special maintenance study. One of these was allowed sufficient food to permit of a gain of ½ pound daily for 6 months (No. 2). Two were kept at constant weight for 6 (No. 3) and 12 months (No. 4). Two others were fed so as to allow a loss of ½ pound per day for 6 (No. 5) and 10 months (No. 6). All the latter animals lost greatly in flesh, especially No. 6 which was in daily danger of dying near the end of the 10 months period. In this case it was clear that the animal had not been receiving sufficient protein in its diet to provide for the considerable skeletal growth which it exhibited and to provide for its maintenance.

Methods of Analysis.

The animals were slaughtered at the end of their experimental periods and a series of weights and measurements was recorded. Various composite and individual samples of the animals were analyzed fresh for water, protein, fat, ash, and phosphorus. Also cold water extracts were made of the flesh, and the nitrogen distribution was studied. The carcasses were hung in coolers for 2 days after slaughter in order to give a good quality of carcass to cut up into the various wholesale cuts. They all lost some moisture in this process which varied from 2.6 to 3.5 per cent of the total weight of the animals (exclusive of the contents of the digestive and excretory tract).

The cold water extracts of the flesh were analyzed for total solids, ash, nitrogen, and phosphorus. The nitrogen coagulated on heating and concentrating (10:1) in the presence of freshly precipitated magnesium carbonate and the nitrogen not precipitated in the cold (15°C.) with 15 per cent sodium chloride and 7.2 per cent tannic acid were also determined. The soluble nitrogen

[3] Trowbridge, P. F., Moulton, C. R., and Haigh, L. D., *Missouri Agric. Exp. Station, Research Bull. 28*, 1918.

could thus be divided into the fraction coagulated on heating, the fraction not coagulated on heating but precipitated with salt and tannic acid, and the fraction not coagulated on heating or on the addition of salt and tannic acid. These fractions will be called albumins, albumoses and peptones, and amino-acids and extractives.

Water extracts of the blood were prepared by shaking the coagulated samples of blood with 10 to 15 gm. of sand and successive small quantities of water and allowing the mixture to settle. The clear liquid was poured off and the process continued until a volume of 500 cc. was obtained. This was heated and concentrated one-half. The resulting filtered solution was treated as was the water extract of the flesh. It still contained some nitrogen coagulable on heating and concentrating (10:1) with magnesium carbonate.

Samples of shoulder meat and the liver of each animal were sent to the laboratory within from 1 to 2 hours after slaughter and the glycogen content was determined by treating the well ground sample with about twice its volume of strong potassium hydroxide solution and digesting. From the resulting solution the glycogen was precipitated by adding an equal volume of 95 per cent alcohol. After it was purified the glycogen was hydrolyzed to dextrose and this was determined by Fehling's solution. For details the original papers must be consulted.[4] The time elapsing between the last meal and slaughtering varied from 2 to 9 hours. The time elapsing between slaughter and the addition of the alkali varied within narrower limits; *i.e.*, from 1 to 2 hours after slaughter.

The methods of analysis were in general those of the Association of Official Agricultural Chemists. Moisture was determined by means of the vacuum desiccator and sulfuric acid. Fat was determined indirectly by ether extraction, nitrogen by the Kjeldahl-Gunning-Arnold method, ash by ignition in porcelain crucibles, and phosphorus on the ashed sample by the gravimetric method. In the water extracts phosphorus was determined after digestion of the evaporated solution with sulfuric acid.

[4] Trowbridge, P. F., and Francis, C. K., *J. Ind. and Eng. Chem.*, 1910, ii, 21, 215.

Selection of Samples and Method of Computing Data.

The presence of stored material in the flesh, such as fat and glycogen, tends to obscure the true relations existing in the tissues. The data are presented on the original wet and fat-containing basis, and to do away with the diluting influence of the fat the data are also shown on the fat-free basis, or on the protoplasmic basis of Greene.[1] I wish to commend this method of computation and to deplore the usual method employed by chemists of calculating to a water- and fat-free basis.

The data are not calculated to the glycogen- and dextrose-free basis because full data on the glycogen and dextrose content are lacking and the amount is comparatively small and not much affected by the state of nutrition. The glycogen content of the shoulder muscle of the extremely emaciated animal (No. 6) was 0.514 per cent on the fat-free basis while a very fat steer of the same age showed 0.459 per cent on the same basis and the control animal (No. 1) had but 0.170 per cent. Since this latter was one of the first animals slaughtered it may well be that the lack of experience with the method of determining glycogen accounts for the low result.

Changes in the Proteins of the Flesh.

Table I presents the data on the changes in composition of the flesh. The water content on the fat-free basis varied from about 76 to 77 per cent. The lower value is shown for a very thin steer as well as for the fat steers. The higher value is found only in the case of the animals whose ration made them grow thinner after having been fat at 11 months. It is not argued, however, that the thinness of the animal is responsible for the higher water content.

The young and old fat steers show between 3.5 and 3.6 per cent of nitrogen in the flesh on the fat-free basis. No. 2 showed a decrease of about 6 per cent, as did the other animals on lower planes of nutrition. The animal subjected to the most extreme treatment (No 6) shows only 3.18 per cent, a decrease from the fat animals of 10 per cent in total nitrogenous matter. This steer has lost 89.5 kilos in body weight, 44.8 kilos of which was lean

TABLE I.

Composition of Composite Flesh.

Animal No.		1	2	3	4	5	6	7
Class		Control.	Supermaintenance.	Maintenance.	Maintenance.	Submaintenance.	Submaintenance.	Full fed.
Condition		Fat.	Fat.	Fairly fat.	Thin.	Very thin.	Very, very thin.	Very fat.
Age		11 mos.	17 mos.	17 mos.	23 mos.	17 mos.	21 mos	21 mos.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water	Fresh.	62.28	60.62	61.89	70.52	71.20	76.37	51.24
	Fat-free.	76.42	75.99	77.05	77.04	75.83	77.04	75.98
Fat, fresh		18.50	20.22	19.67	8.47	6.10	1.87	32.55
Solids	Fresh.	37.71	39.37	38.10	29.48	28.79	23.63	48.75
	Fat-free.	23.57	24.00	22.94	22.95	24.17	22.96	24.01
Water-soluble solids	Fresh.	4.35	3.73	4.12	4.69	4.85	4.21	3.47
	Fat-free.	5.33	4.68	5.13	5.12	5.16	4.29	5.15
Ash	Fresh.	0.852	0.805	0.798	0.956	0.888	1.045	0.703
	Fat-free.	1.045	1.009	0.993	1.044	0.946	1.054	1.042
Water-soluble ash	Fresh.	0.766	0.493	0.647	0.709	0.776	0.890	0.604
	Fat-free.	0.940	0.618	0.805	0.775	0.826	0.907	0.896
Phosphorus	Fresh.	0.167	0.160	0.151	0.186	0.178	0.174	0.133
	Fat-free.	0.205	0.201	0.188	0.203	0.190	0.176	0.197
Water-soluble phosphorus	Fresh.	0.127	0.119	0.117	0.143	0.141	0.128	0.089
	Fat-free.	0.156	0.149	0.146	0.156	0.150	0.130	0.132
Nitrogen	Fresh.	2.922	2.632	2.700	3.082	3.139	3.150	2.384
	Fat-free.	3.585	3.299	3.361	3.367	3.343	3.180	3.535
Water-soluble nitrogen	Fresh.	0.524	0.451	0.497	0.526	0.585	0.463	0.148
	Fat-free.	0.643	0.565	0.619	0.575	0.623	0.467	0.620
Insoluble nitrogen, fat-free		2.942	2.734	2.742	2.792	2.720	2.713	2.915
Albumin nitrogen	Fresh.	0.239	0.176	0.195	0.240	0.262	0.152	0.156
	Fat-free.	0.293	0.221	0.243	0.262	0.279	0.153	0.231
Albumose peptone nitrogen	Fresh.	0.100	0.106	0.123	0.084	0.112	0.108	0.099
	Fat-free.	0.123	0.132	0.153	0.092	0.133	0.109	0.147
Amino-acid extractive nitrogen	Fresh.	0.185	0.169	0.179	0.202	0.211	0.203	0.163
	Fat-free.	0.227	0.212	0.223	0.221	0.225	0.205	0.242

flesh and 10.9 kilos of which was protein. Besides this loss the animal suffered a depletion in the richness of the remaining nitrogenous material of 10 per cent.

It is of interest to note what classes of nitrogenous matter contributed to this loss. In most of the thin steers the insoluble nitrogenous matter accounts for all the loss, but in the case of No. 6 it accounts for only part of the loss. The remaining loss was in the soluble nitrogen and especially the albumins which showed a

TABLE II.

Nitrogen Distribution in Composite Flesh.

Animal No.	1	2	3	4	5	6	7
Class	Control.	Super-maintenance.	Maintenance.	Maintenance.	Sub-maintenance.	Sub-maintenance.	Full fed.
Condition	Fat.	Fat.	Fairly fat.	Thin.	Very thin.	Very, very thin.	Very fat.
Age	11 mos.	17 mos.	17 mos.	23 mos.	17 mos.	21 mos.	21 mos.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water-soluble N	17.93	17.12	18.41	17.07	18.63	14.68	17.53
Water-insoluble N	82.06	82.87	81.58	82.92	81.36	85.31	82.46
Albumin N	8.17	6.69	7.23	7.78	8.34	4.81	6.53
Albumose and peptone N	3.43	4.00	4.55	2.73	3.56	3.42	4.15
Amino-acid and extractive N	6.33	6.42	6.63	6.56	6.73	6.44	6.84
Amino-acid and extractive nitrogen per 100 gm. water.							
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
	297	279	289	287	297	266	318

loss from the control animal (No. 1) of 28 per cent. No. 6 also showed a loss of 10 per cent in the amino-acid and extractive nitrogen from the control (No. 1) or 18 per cent from No. 7.

The distribution of nitrogenous material in percentage of the total nitrogenous material is shown in Table II. It is seen that the water-soluble and water-insoluble nitrogen remain rather constant in all cases but No. 6 which exhibits a decrease of 4 per cent of the total in this fraction, all of which is due to the albumin fraction. The amino-acid and extractive nitrogen remains prac-

tically constant in all cases. The increase that is shown is believed to be due to increasing age. The flesh of older steers runs higher in flavor; *i.e.*, in the extractives. The concentration of the amino-acids and extractives per 100 gm. of water remains about constant except for No. 6 which shows a 10 per cent decrease from No. 1 or about 18 per cent decrease from No. 7.

The Other Constituents of the Flesh.

On the whole the total ash and water-soluble ash remain about constant on the fat-free basis. The total phosphorus is rather constant excepting for No. 6 which shows about a 13 per cent decrease. The same is true of the water-soluble phosphorus where No. 6 shows a decrease of about 15 per cent. No. 7 also shows a lowered value here while the 23 months old thin steer (No. 4) shows a maximum.

Changes in Composition of the Liver.

The ether extract (fat) of the liver varies as a rule between rather narrow limits. So the data shown in Table III are interpreted the same on either basis. The liver of No. 6 has the highest water content and the lowest content of solids. In spite of this it exhibits the highest nitrogen and ash content. The phosphorus content is slightly low in several of the steers but not in either of the fat steers. With a lowered content of solids in the liver of No. 6 there must be a lowered protein content and so the increased nitrogen content must be due to a larger proportion of products of catabolism of higher nitrogen content such as is shown by the nitrogenous extractives. It is unfortunate that a separation of the nitrogenous bodies of the liver had not been made. Perhaps too much emphasis had better not be laid on this apparent change in nitrogenous matter since the high value for nitrogen is the result of the average of the triplicate results, one of which was high. If the two nearest results are averaged the nitrogen content is normal. This point needs verification.

The glycogen content of the liver is of interest in this connection. In the control animal there was 0.601 per cent glycogen on the fresh basis. The fairly fat maintenance steer (No. 3) had 0.965

per cent, No. 6 0.913 per cent, and No. 7 0.884 per cent. All these differences might well be due to differences in time which elapsed between feeding and slaughter and between slaughter and analysis of the sample.

TABLE III.

Composition of Liver.

Animal No.		1	2	3	4	5	6	7
Class		Control.	Super-maintenance.	Maintenance.	Maintenance.	Sub-maintenance.	Sub-maintenance.	Full fed.
Condition		Fat.	Fat.	Fairly fat.	Thin.	Very thin.	Very, very thin.	Very fat.
Age		11 mos.	17 mos.	17 mos.	23 mos.	17 mos.	21 mos.	21 mos.
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water	Fresh.	68.82	70.34	70.30	70.85	69.68	71.34	69.22
	Fat-free.	72.64	71.94	72.24	73.31	71.22	74.11	70.94
Fat, fresh		5.26	2.22	2.69	3.36	2.17	3.73	2.43
Solids	Fresh.	31.17	29.65	29.70	29.15	30.32	28.66	30.78
	Fat-free.	29.35	28.05	27.75	26.68	28.77	25.88	29.05
Nitrogen	Fresh.	3.06	2.97	2.95	3.19	3.49	3.65*	3.27
	Fat-free.	3.23	3.04	3.03	3.30	3.59	3.79*	3.35
Ash	Fresh.	1.340	1.443	1.376	1.473	1.453	1.540	1.352
	Fat-free.	1.414	1.476	1.414	1.524	1.485	1.600	1.386
Phosphorus	Fresh.	0.347	0.336	0.332	0.319	0.386	0.333	0.391
	Fat-free.	0.366	0.344	0.341	0.330	0.395	0.346	0.410

* Average of triplicate results. When one high result is discarded these figures become 3.283 and 3.411, respectively.

Changes in Composition of the Blood.

Table IV shows the composition of the blood of the animals.

The water content of the blood varies with the fat content of the steer being higher in the thinner steers and lower in the fatter ones. A poor state of nutrition seems to give a "thinner" blood. No. 6 had 5 per cent more water in the blood than did No. 7.

Two of the steers show a very high ash content, the thin maintenance animal and the fasted steer (Nos. 4 and 6). Too much emphasis should not be placed upon this until it is confirmed by further work. The phosphorus content is lowest in No. 6. It is, however, fairly constant throughout for all animals but the control animal. The high result here may be an error since in the analysis of the blood from thirty-five animals only two had more than 0.030 per cent of phosphorus.

TABLE IV.

Composition of Blood.

Animal No.	1	2	3	4	5	6	7
Class	Control.	Supermaintenance.	Maintenance.	Maintenance.	Submaintenance.	Submaintenance.	Full fed.
Condition	Fat.	Fat.	Fairly fat.	Thin.	Very thin.	Very, very thin.	Very fat.
Age	11 mos.	17 mos.	17 mos.	23 mos.	17 mos.	21 mos.	21 mos.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Water	79.34	80.57	80.60	80.63	81.20	83.68	78.65
Ash	0.318	0.358	0.339	0.747	0.322	0.800	0.387
Phosphorus	0.055	0.025	0.025	0.029	0.024	0.019	0.022
Total nitrogen	3.182	3.002	2.987	3.008	2.897	3.135	3.334
Water-soluble nitrogen	0.295	0.785	0.568	0.400	0.556	0.381	0.503
Albumin nitrogen	0.048	0.434	0.338	0.040	0.257	0.086	0.332
Albumose and peptone nitrogen	0.186	0.288	0.157	0.239	0.176	0.263	−0.032
Amino-acid and extractive nitrogen	0.061	0.063	0.073	0.121	0.123	0.032	0.203
Ratio N:P	57.9	120.1	119.5	103.7	120.7	165.0	152.0

The nitrogen content of the blood varies within rather narrow limits. The very fat steer (No. 7) had somewhat the highest content of nitrogen and the lowest is shown by the very thin submaintenance steer (No. 5). The results of the separation of nitrogenous bodies are unsatisfactory. The method employed gave rather large variations in the triplicate determinations and a lack of uniformity in the amount of coagulable nitrogen remaining in solution after the first heating. No attempt will be made to interpret the results shown. The method used should be improved or discarded.

The ratio of nitrogen to phosphorus in the blood is shown in Table IV. Age seems to play a rôle in the change of ratio. No. 1 had a ratio of nitrogen to phosphorus of 57.9. The 17 months steers had about 120 irrespective of condition or plane of nutrition. The abnormally high phosphorus content of the control steer's blood is, perhaps, responsible for the low ratio of 57.9. With the animals 21 to 23 months old this ratio is very high in the case of both the very fat and the emaciated steer, about 160. The third steer shows a low value of about 104. Neither the condition of the steer nor the plane of nutrition seems to be responsible for the change.

Changes in the Size of the Muscle Fibers.

It has been shown that underfeeding decreased the weight of lean flesh about 44.8 kilos in an animal weighing about 300 kilos. The total loss of protein, calculated from the nitrogen content, was 10.9 kilos. The quality of the protein had been altered by a great decrease in the albumin fraction. Fig. 1 shows the changes in the muscle fibers that accompanied these changes. The fiber designated as "very thin" was from the biceps muscle of the fasted steer (No. 6). The other fibers were from the same muscles of others of the animals. The figure has been published in another connection[5] but is shown again here with some added data.

There is little difference in the diameter of muscle fibers of very fat or thin steers, the former having a diameter of 50 micra and the latter 45 micra. No. 6, however, had fibers that had shrunk to 20 micra in diameter, but 40 per cent of the original diameter. The area of the cross section will vary as the square of the radius, or as 625:506:100. The cross section of No. 6's biceps fiber was one-fifth as great as that of a thin steer and one-sixth as great as that of a fat steer.

The length of the segments of the muscle fibers shows a similar decrease. For the fat steer the average length is 14.4 micra, for the thin steer 10.9 micra, and for the fasted steer 6.4 micra. This would argue that the muscle fiber had decreased over one-half in length.

[5] Waters, H. J., *Proc. 30th Meeting Soc. Promotion of Agric. Sc.*, 1909.

The volume of a segment is obtained from the formula for the volume of a cylinder, $\pi r^2 h$, where r is the radius of the fiber and h is the average length of the segment. In round numbers the relative volume of an average segment was 9,000 for the fat steer, 5,500 for the thin steer, and but 640 for the fasted steer. The

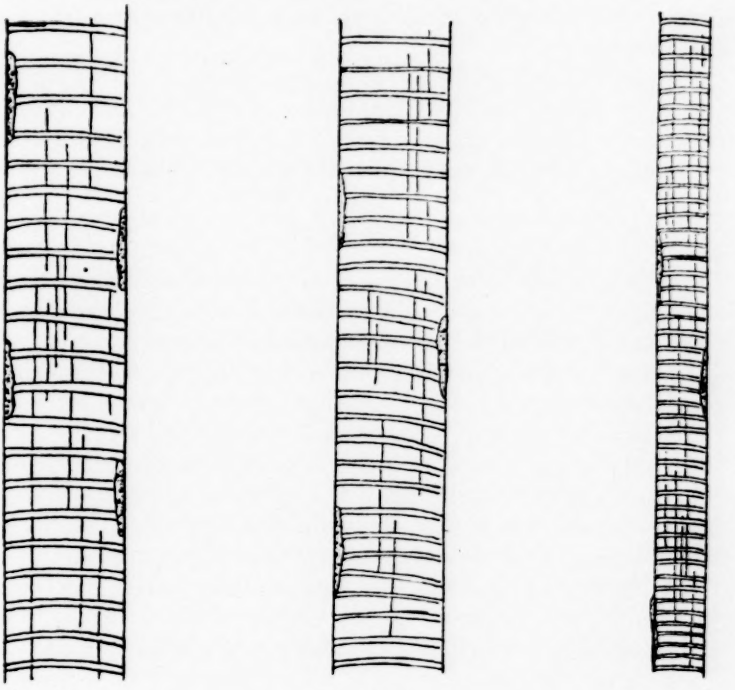

FIG. 1. The size of muscle fiber as influenced by underfeeding.

ratios are then 14.0:8.6:1. If this is any proper index of muscle volume the possibilities for change in size or volume are stupendous.

No evidence was found in this study of a disintegration of muscle fibers. The very thin fiber still functioned as a muscle fiber and it would appear that its vital structure must be intact. The

change therefore must be due to a resorption of inter- and intracellular substances of a stored nature. Part of this no doubt was fat, but considerable of it was nitrogenous matter, as shown above.

It must be remembered that the thin and fasted steers had previously been as fat as the control steer and presumably had the same make-up of nitrogenous matter and the same size of muscle fibers. The change is one of resorption and stored protein has been removed.

A better histological picture of the muscle fibers would contribute materially to the force of the arguments presented. It is to be regretted that the figure is diagrammatic although based upon averaged observations made upon the muscle fibers of the steers.

SUMMARY.

Inanition or partial starvation does not cause a watery muscular tissue. Fat is almost entirely resorbed while glycogen apparently is not. The character of the protein is altered by a removal of nitrogenous substances amounting to 10 per cent of the total nitrogenous material and consisting of the albumin nitrogen. The phosphorus content of the flesh is reduced 13 to 15 per cent.

The liver has a somewhat higher water content in the starved animal accompanied by a high nitrogen content. This would indicate a greater proportion of amino-acid and extractive nitrogenous substances. The glycogen content is not depleted.

The blood has more water and less total nitrogen in the fasted steer.

The muscle fibers have become very much smaller, but still are functioning muscle fibers.

It is believed that the data presented indicate a storage of protein matter in the muscles of the beef steer.

AMINO-ACID SYNTHESIS IN THE ANIMAL ORGANISM.

CAN NOR-LEUCINE REPLACE LYSINE FOR THE NUTRITIVE REQUIREMENTS OF THE WHITE RAT?

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Of the possibility of amino-acid synthesis in the normal mammalian organism, little is definitely known. Perfusion experiments with the surviving liver (1) have shown that α-keto- and α-hydroxy-acids may be converted to the corresponding amino-acids, to the optical isomer that exists in the protein molecule. The formation of tyrosine, phenylalanine, alanine, leucine, and nor-leucine among others has thus been demonstrated. In the living organism, however, the synthesis of α-amino-acids by the reaction between ammonia and non-nitrogenous rests cannot be regarded as proved, despite the many experiments (2) which have been undertaken with this in view. Of amino-acids present in the protein molecule, only glycocoll is known to be synthesized, and even in the case of this amino-acid no proof exists as to whether it takes its origin from glycollic or glyoxylic acid and ammonia, a direct synthesis of the type discussed above, or whether it arises from cleavage of some other amino-acid of higher molecular weight (3). Tyrosine may possibly be derived from phenylalanine (4), and histidine from arginine (5), but neither of these reactions involves an amination of a non-nitrogenous carbon chain.

The present study is concerned with the formation of lysine by amination of the ϵ carbon of nor-leucine. It seemed possible that, if the α-amino group were already present in the molecule, further substitution of a second amino group might take place more readily. The possibility also suggested itself that, even if this formation of lysine from nor-leucine did not occur, nor-leucine might

still have a value in nutrition. According to the current idea, the ϵ group of lysine does not function in peptide synthesis and is present in the protein molecule free and not combined in the peptide linkage. The organism might have the power to synthesize a new type of protein under stress by the substitution of nor-leucine for lysine, a protein which would contain little or no free amino nitrogen, since it is to the ϵ group of lysine that this free amino nitrogen of the protein molecule is usually attributed.

The validity of the present experimental work is dependent upon the fact that lysine in adequate amounts is essential for the normal growth of the young white rat. Osborne and Mendel (6) have shown that gliadin with its low content of lysine as the sole source of the protein of the diet does not permit normal growth unless supplemented by lysine, while with zein (7), which is deficient in both tryptophane and lysine, as the source of protein maintenance results from the addition of tryptophane, but no growth unless lysine is also added. Hart, Nelson, and Pitz (8) were unable to demonstrate a synthesis of lysine by the mammary gland of the white rat, to supply an adequate milk for the young. Buckner, Nollau, and Kastle (9), and Osborne and Mendel (10) have also demonstrated that chickens require a sufficient amount of lysine to make normal growth.

Nor-leucine, the third of the isomeric α-amino-caproic acids known to exist in the protein molecule, was discovered by Abderhalden and Weil (11) in 1912 in the proteins of the central nervous system. However, it is in all probability also present in proteins derived from other sources, but since its solubilities and general properties so closely resemble those of the other isomeric α-amino-caproic acids as to make its separation from them difficult, its occurrence has as yet been reported in the proteins of the nervous system only. Greenwald (12) has demonstrated that nor-leucine behaves normally in the animal body, yielding its nitrogen as urea after ingestion. He has also observed that nor-leucine is a glucose-former in the phlorhizinized dog, and that the amount of glucose formed from the *d*, *l*, or *dl* forms does not vary appreciably.

EXPERIMENTAL.

Young white rats of 50 to 60 gm. weight for the most part were used as experimental animals. They were kept in individual cages which permitted accurate record of the food consumption. The usual precautions to insure cleanliness and prevent infection were taken. It may be noted that only one case of disease, a type of pulmonary infection, appeared in the colony. The food which was high in fat content was made into small balls weighing 5 to 10 gm. each which were kept in an ice chest until used. In this form the food was not easily scattered by the animal and there was little wasting.

The casein, gliadin, nor-leucine, and lysine were prepared in the laboratory. The lysine was obtained by hydrolysis of casein, while the nor-leucine was a synthetic product. The protein-free milk prepared by the method of Osborne and Mendel contained 0.59 per cent nitrogen. There has been considerable criticism of the use of this substance in nutrition experiments from time to time, but in the present series the objections offered seemed to be invalid, since the diet as a whole remained constant throughout with variations from the addition of small quantities of the amino-acids only. If there were any appreciable amounts of essential amino-acids furnished by the protein-free milk, these were present in each of the periods, and their influence should have been uniform.

The experimental dietaries were as follows:

	per cent		*per cent*
Casein or gliadin	18.0	Amino-acid	0.5
Protein-free milk	28.0	Gliadin	17.5
Corn-starch	24.0	Protein-free milk	28.0
Purified butter fat	18.0	Corn-starch	22.0
Lard	12.0	Purified butter fat	18.0
	100.0	Lard	14.0
			100.0

Rats were maintained on diets containing 18 per cent gliadin for periods of several weeks; nor-leucine replaced part of the gliadin usually in amounts equivalent to 0.5 per cent of the diet, the protein plus amino-acid still constituting 18 per cent of the total ration; finally in some experiments equivalent amounts of lysine

replaced the nor-leucine. The relative order of these periods was varied, but the general procedure remained the same. Controls were fed on casein rations to demonstrate that normal growth was possible on these diets provided the protein was adequate.

In some of the experiments, 1.5 per cent of nor-leucine was fed instead of 0.5 per cent. Lysine to the extent of 0.5 per cent will supplement a diet containing 18 per cent gliadin such as was used in these experiments (6) and allow normal growth. The nor-leucine, however, was racemic and as it was considered desirable to insure an adequate amount of the *d* form in the diet, in case the animal could not use both forms equally well, the higher percentages were added to the ration. In later experiments through the kindness of Dr. C. S. Marvel of this laboratory, we were supplied with small amounts of *d*-nor-leucine, which showed on analysis 82 per cent of the *d* form. This was added to the diet to the extent of 0.658 per cent equivalent to 0.5 per cent of the *d* form. No difference could be observed between this optically active form and the racemic compound.

DISCUSSION.

The results of the experiments are given in Table I and Charts 1 and 2. Rats fed on casein grew normally in all cases.[1] The curves for Rats 5, 8, and 17 show the type of growth obtained when gliadin was substituted for casein. Slight growth ensued, a result which was in agreement with the findings of Osborne and Mendel (6) previously discussed. Rat 3 was fed a diet in which 0.5 per cent *dl*-nor-leucine replaced an equivalent amount of gliadin. The curve of growth for this animal closely resembles that for the rats fed gliadin alone. Animals which received a higher percentage of the *dl*-nor-leucine (Rats 6, 8, 14, 17) did not make any better growth than those fed 0.5 per cent. The cessation of growth in older animals, which had been gaining in weight steadily on casein diets, when gliadin plus nor-leucine was substituted as the source of protein may be observed in the curves for Rats 11 and 12. In order to prove that this failure to increase in weight on gliadin and nor-leucine was not due to the

[1] The growth curves of the casein-fed controls are not shown in the charts, but Table I includes a record of their weights and food consumption.

TABLE I.

Summary of Weights and Food Intake of Experimental Animals.

Rat No	Diet.	Duration	Initial weight.	Final weight.	Gain.	Total food.	Food per 100 gm. of rat.	Gain per 100 gm. of food
		days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1 ♂	Casein.	98	97	250	152	877	32	17.8
9 ♂	"	84	63	190	127	616	37	20.5
16 ♂	"	56	62	206	144	453	40	31.9
2 ♂	Gliadin.	91	100	117	16	456	34	3.7
13 ♀	"	91	68	88	20	451	50	4.3
3 ♂	(a) Gliadin.	28	101	101	00	152	37	0.0
	(b) " + 0.5 per cent *dl*-nor-leucine.	91	101	132	30	496	31	4.0
11 ♂	(a) Casein.	42	70	150	80	296	43	27.0
	(b) Gliadin + 0.5 per cent *dl*-nor-leucine.	91	150	149	−1.3	610	31	−0.2
12 ♀	(a) Casein.	42	70	130	60	275	44	22.0
	(b) Gliadin + 1.5 per cent *dl*-nor-leucine.	98	130	140	10	558	30	1.7
5 ♀	(a) Gliadin.	70	49	58	10	271	48	3.5
	(b) " + 1 per cent lysine.	42	58	113	54	243	40	22.2
	(c) Gliadin + 0.5 per cent *dl*-nor-leucine.	35	113	115	2.3	173	25	1.3
8 ♂	(a) Gliadin.	42	76	77	1	184	36	0.5
	(b) " + 1.5 per cent *dl*-nor-leucine.	84	77	88	11	349	33	3.1
	(c) Gliadin + 0.5 per cent *d*-nor-leucine.	42	88	91	3	171	31	1.7
	(d) Gliadin + 0.5 per cent lysine.	42	91	120	19	208	31	9.3
6 ♀	(a) Gliadin.	28	63	62	−1	133	43	−0.7
	(b) " + 1.5 per cent *dl*-nor-leucine.	56	62	74	11	230	38	4.8
	(c) Gliadin + 1 per cent lysine.	42	74	123	50	248	35	20.0
	(d) Gliadin + 0.5 per cent *dl*-nor-leucine.	35	123	126	2	205	28	1.2

TABLE I—*Concluded.*

Rat No.	Diet.	Duration.	Initial weight.	Final weight.	Gain.	Total food.	Food per 100 gm. of rat.	Gain per 100 gm. of food.
		days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
4 ♂	(a) Casein.	28	59	106	47	140	35	33.7
	(b) Gliadin + 0.5 per cent *dl*-nor-leucine.	63	106	118	12	397	34	3.0
	(c) Gliadin + 0.5 per cent lysine.	35	118	167	49	249	29	15.5
	(d) Gliadin + 1.5 per cent *dl*-nor-leucine.	35	167	160	97	234	24	−3.0
14 ♂	(a) Gliadin + 1.5 per cent *dl*-nor-leucine.	84	62	62	00	281	35	00.0
	(b) Gliadin + 0.5 per cent lysine.	35	62	97	35	143	35	24.4
15 ♀	(a) Gliadin + 1.5 per cent *dl*-nor-leucine.	56	57	57	00	199	44	00.0
	(b) Gliadin + 0.5 per cent *d*-nor-leucine.	42	57	65	8	110	30	7.2
17 ♀	(a) Gliadin.	56	57	59	2	224	50	0.9
	(b) " + 1.5 per cent *dl*-nor-leucine.	63	59	62	3	185	34	1.6

inability of the organism to resolve and metabolize the racemic amino-acid, Rats 8 and 15 were fed *d*-nor-leucine in place of the racemic form. The results differed in no way from those of the preceding experiments.

As a final check on the experimental procedures, lysine replaced the nor-leucine of the diet of Rats 4, 5, 6, 8, and 14 (Chart 2). In confirmation of the work of Osborne and Mendel, this amino-acid supplemented the diet in such a way as to permit normal growth. On the addition of lysine, there was a marked improvement in the general condition of the rats previously stunted by a gliadin or a gliadin plus nor-leucine diet, an improvement which was noticeable within the 1st week.

The increased rate of growth is not to be attributed to an increased food consumption, since as shown in the table the food eaten per 100 gm. of body weight in the lysine period was practically the same as in the preceding nor-leucine period. The gains per 100 gm. of food consumed on the lysine-gliadin diets were com-

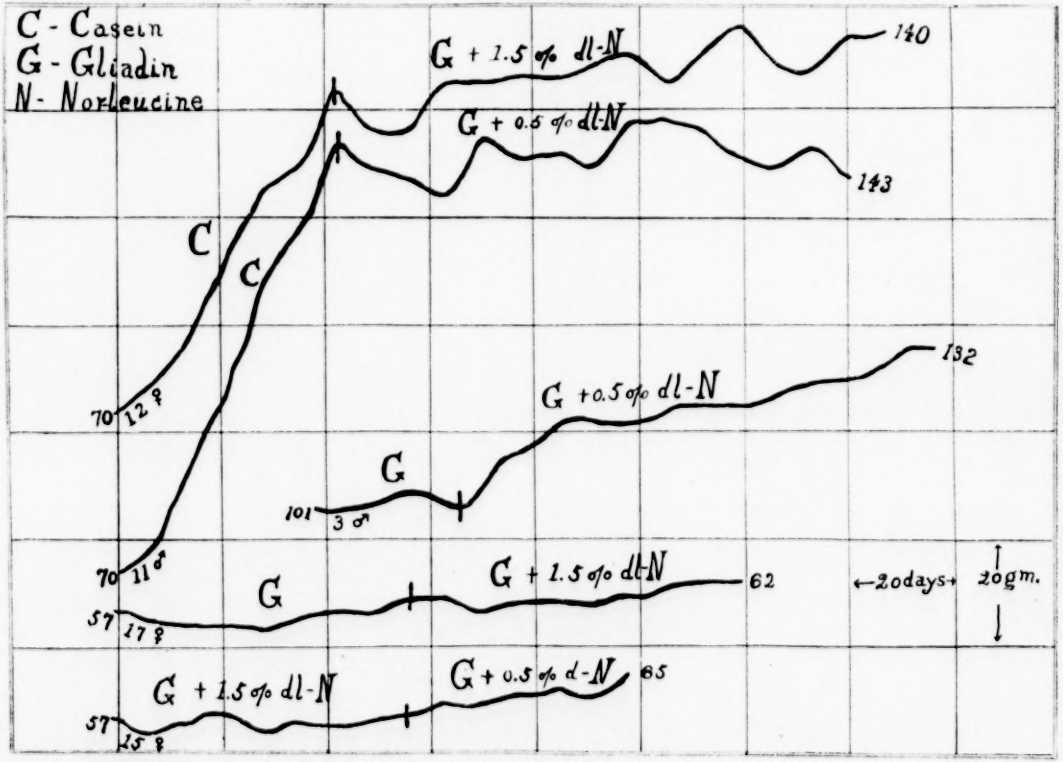

CHART 1. Showing the failure to grow normally when *dl*-nor-leucine supplements an 18 per cent gliadin diet. The curves for Rats 11 and 12 also show the prompt cessation of growth in older and larger rats when casein is replaced by gliadin and nor-leucine. In the case of Rat 15 no improvement results from the ingestion of the *d*-nor-leucine in place of the *dl* form.

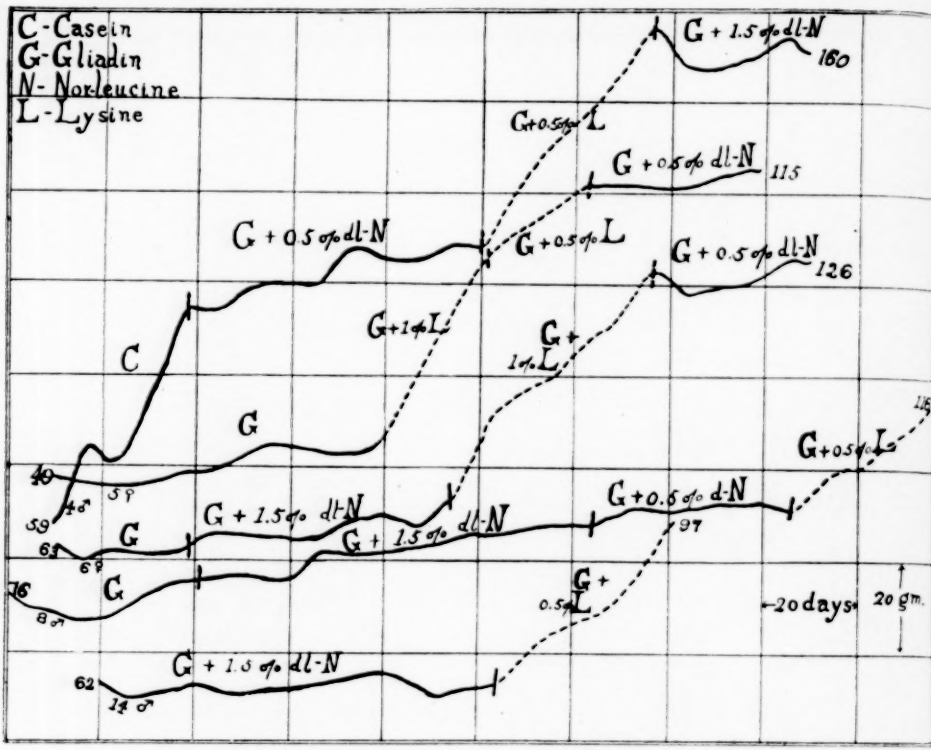

CHART 2. Showing that the deficiency in a gliadin plus nor-leucine diet may be remedied by substitution of lysine (dotted line) for nor-leucine, and that cessation of growth ensues when nor-leucine replaces lysine. The curve for Rat 8 also demonstrates that *d*-nor-leucine is no more efficient as a supplement to gliadin than is the racemic compound.

parable to those made on the casein diets, while with gliadin or gliadin plus nor-leucine the gains in proportion to the food consumed were almost negligible.

SUMMARY.

1. Gliadin comprising 18 per cent of the diet has been shown to be inadequate for normal growth of the white rat, and to permit of maintenance or slow growth only. Lysine in amounts equiva-

lent to approximately 3 per cent of the protein of the above diet without further alteration renders the diet adequate for normal growth. These results are in agreement with those of Osborne and Mendel.

2. Neither *dl*- nor *d*-nor-leucine is able to supply the deficiency of a gliadin diet as does lysine. When nor-leucine is replaced by lysine in the experimental dietaries, normal growth ensues.

3. No evidence has been obtained which would indicate that nor-leucine is a precursor of lysine or that the organism of the white rat can substitute nor-leucine for lysine in the synthesis of its body protein for growth.

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EFFECT OF HYPODERMIC AND ORAL ADMINISTRATION OF CALCIUM SALTS ON THE CALCIUM CONTENT OF RABBIT BLOOD.

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Since the appearance of the work of Weiske (1), Forster (2), and Voit (3), who were among the first to realize clearly the importance of calcium for growth and maintenance, numerous attempts have been made to determine the various factors involved in the metabolism of this element. Although we know that it is possible, under certain dietary conditions, to cause an animal to store considerable amounts of calcium, no definite site of storage has been demonstrated. Weiske (1), Voit (3), Aron and Sebauer (4), Kost (5), Patterson (6), and Denis and Minot (7), experimenting with various animals on calcium-rich and calcium-poor diets, were unable to demonstrate any appreciable differences in the calcium content of the blood, bones, or tissues.

The experimental work[1] presented in this paper was carried out to ascertain if it is possible, by various means, to produce a definite increase in the calcium content of the circulating blood of different animals and, if so, whether the increase is permanent or transitory. The following methods were used in an attempt to increase the calcium content of the blood.

1. Intravenous and subcutaneous injections of calcium salts.
2. A calcium-rich diet.

Two groups of experiments were carried out to determine the effects of injected calcium salts. In the first, rabbits confined in metabolism cages were fed a diet of equal amounts of rolled

[1] This work was begun in 1917, with H. A. Mattill, Department of Nutrition, University of California, but was soon suspended and not taken up again until late in 1918.

TABLE I.

Effects of Intravenous and Subcutaneous Injections of Calcium Salts.

Day of experiment.	Ca per 100 cc. of whole blood.*	Remarks.	Weekly calcium.			
			Ingested.†	Eliminated.†		Balance.
				Urine.	Feces.	
		Rabbit 1 ♀, weight 2,640 gm.				
	mg.		*gm.*	*gm.*	*gm.*	*gm.*
7	10.81					
10	10.10					
14	11.75	40 min. after intravenous injection of 63 mg. of Ca, as 1 per cent calcium chloride.	0.678	0.070	0.937	−0.329
14	9.87	130 min. after above injection.				
14	8.93	235 " " " "				
21	8.84	193 " " subcutaneous injection of 38 mg. of Ca, as saturated calcium hydroxide.	0.498	0.034	0.675	−0.211
29	9.12	5 min. after subcutaneous injection of 19 mg. of Ca, as saturated calcium hydroxide.	0.686	0.030	0.609	+0.047
		Rabbit 2 ♀, weight 3,285 gm.				
10	13.58					
12	12.22					
14	13.16	75 min. after subcutaneous injection of 63 mg. of Ca, as 1 per cent calcium chloride.	0.248	0.230	0.363	−0.345
14	12.69	180 min. after above injection.				
21	11.84	15 " " subcutaneous injection of 38 mg. of Ca, as saturated calcium hydroxide.	0.391	0.181	0.335	−0.125
21	11.84	75 min. after above injection.				
21	11.00	260 " " " "				
29	12.31	7 " " subcutaneous injection of 17 mg. of Ca, as saturated calcium hydroxide.	0.579	0.203	0.834	−0.458
29	13.07	30 min. after above injection.				

* Calcium determinations made by author's method (unpublished work), which, with modifications, is the same as that outlined by Halverson and Bergeim (8).

† Calcium determinations in food, feces, and urine made by McCrudden's (9) method.

barley and fresh carrots, practically a balanced ration with respect to its acid- and base-forming constituents.[2] After a normal period of 14 days the animals received weekly injections of calcium salts, in doses varying from 19 to 63 mg. of Ca. In the second group, the animals were fed a normal diet (alfalfa, barley, greens), and were given much larger injections, 200 to 250 mg. of Ca every 5th day. The results of these experiments, given in Tables I, II, and III, indicate a rapid elimination of the injected calcium. Although most of the calcium, 80 to 90 per

TABLE II.

Effects of Intravenous and Subcutaneous Injections of Calcium Salts.

Rabbit 3 ♂, weight 3,263 gm.

Day of experiment.	Ca per 100 cc. of whole blood.*	Remarks.
	mg.	
7	10.78	
9	10.81	
11	9.40	
14	11.28	70 min. after the intravenous injection of 63 mg. of Ca, as 1 per cent calcium chloride.
14	8.93	185 min. after above injection.
14	8.93	315 " " " "
21	12.50	45 " " the subcutaneous injection of 50 mg. of Ca, as saturated calcium hydroxide.

* Calcium determinations made by author's method (unpublished work), which, with modifications, is the same as that outlined by Halverson and Bergeim (8).

cent, is rapidly eliminated, it is possible to obtain a definite but transitory increase in the calcium content of the blood, this being in agreement with the work of Heubner and Rona (11) who used a large number of cats as experimental animals and with that of von Fenyvessy and Freund (12) who used rabbits.

One rabbit was used as a control to determine the effects of frequent hemorrhage upon the calcium content of whole blood and plasma. Approximately the same amounts of blood were taken from this animal at time intervals corresponding to the

[2] According to data given by Sherman and Gettler (10).

removal of blood samples from the animals receiving injections of calcium salts. The results, given in Table IV, show that repeated hemorrhage causes a decrease, 10.7 to 12 per cent, in the calcium content of the plasma but only a slight and questionable change in the whole blood. The same effects are observed in the animals receiving the injections of calcium salts. Heubner

TABLE III.

Effects of Intravenous Injections of Calcium Chloride.

Day of experiment.	Hematocrit.*	Ca per 100 cc. of whole blood.†	Ca per 100 cc. of plasma.†	Remarks.
Rabbit 4 ♂, weight 3,636 gm.				
	per cent	*mg.*	*mg.*	
1	38.7	9.58	12.14	
4	39.5	10.15		
5	37.4	10.53	13.71	
5	33.1	10.53	14.56	120 min. after intravenous injection of 200 mg. of Ca, as 0.5 N calcium chloride.
5	33.4	9.71	12.99	195 min. after above injection.
10	30.9	10.68		
10	27.1	10.83	14.38	180 min. after intravenous injection of 250 mg. of Ca, as 0.5 N calcium chloride.
10	27.4	10.05	13.66	300 min. after above injection.
Rabbit 5 ♂, weight 3,523 gm.				
4	40.2	9.33	13.62	
5	36.3	8.81	12.95	
5	35.3	11.05	15.75	70 min. after intravenous injection of 200 mg. of Ca, as 0.5 N calcium chloride.
5	31.1	7.54	11.42	240 min. after above injection.
5	29.6	8.21	10.66	340 " " " "
10	31.8	9.63	13.37	
10	31.4	10.05	15.23	120 min. after intravenous injection of 250 mg. of Ca, as 0.5 N calcium chloride.
10	27.2	8.96	11.95	240 min. after above injection.
10	27.6	8.89	11.79	360 " " " "

* Hematocrit values obtained by centrifuging in capillary tubes for 10 min. at approximately 3,600 revolutions per min.

† Calcium determinations in whole blood and plasma made by author's (13) method of direct precipitation.

and Rona (11) report somewhat similar results but found that both plasma and whole blood gave normal calcium values by the end of the 7th hour. The data presented in this paper, contrary to the report of the above authors, does not show any tendency towards a return to normal at the end of the 5th or 6th hour.

The effects of a calcium-rich diet on the calcium content of the blood are given in Table V. The diet was a mixture of four parts of rolled barley and one part of alfalfa leaves containing 2 per cent of calcium lactate. The drinking water also contained 2 per

TABLE IV.

Effects of Frequent Hemorrhage on the Calcium Content of Whole Blood and Plasma.

Rabbit 6 ♂, weight 2,500 gm.

Day of experiment.	Hematocrit.*	Ca per 100 cc. of whole blood.†	Ca per 100 cc. of plasma.†	Remarks.
	per cent	*mg.*	*mg*	
4	36 6	9.78	12 84	
5	32.2	9.59	12.92	Blood sample at 10.50 a.m.
5	29.1	9.11	12.32	70 min. later.
5	30.9	9.11	11.27	250 " "
10	33 7	9.03	12.47	Blood sample at 9.30 a.m.
10	29 4	8.89	12.02	120 min. later.
10	29.1	8.66	12.32	240 " "
10	27.3	8.58	11.13	360 " "

* Hematocrit values obtained by centrifuging in capillary tubes for 10 min. at approximately 3,600 revolutions per min.

† Calcium determinations in whole blood and plasma made by author's (19) method of direct precipitation.

cent of calcium lactate. This was the only calcium salt that seemed at all acceptable to the animals and even this resulted in a much diminished food intake. The high calcium content of the alfalfa leaves (a composite sample gave 2.12 per cent Ca[3] on a dry basis), and the readiness with which they are ingested would indicate a more successful means of increasing the calcium intake. The results of these experiments show that it is not possible to increase the calcium content of rabbit blood by feeding a calcium-rich diet, which is in agreement with the recent work of

[3] Forbes (14) reports 2.14 per cent Ca in alfalfa leaves.

Denis and Minot (7) who carried out similar experiments with men, rabbits, and dogs. Goitein (15) and Voorhoeve (16) report definite increases in bone and blood calcium after the ingestion of a calcium-rich diet. The analytical methods used by the latter are of such a nature as to make the results of doubtful value.

TABLE V.

Effects of a Calcium-Rich Diet on the Calcium Content of Blood and Plasma.

Day of experiment.	Hematocrit.*	Ca per 100 cc. of plasma.†	Ca per 100 cc. of whole blood.†	Weekly calcium.			
				Ingested.‡	Eliminated.‡		Balance.
					Urine.	Feces.	
Rabbit 7 ♂, weight 3,436 gm.							
	per cent.	*mg.*	*mg.*	*gm.*	*gm.*	*gm.*	*gm.*
Control period.							
1	31.1	12.93					
30	36.0	13.04					
41		11.55					
43	32.5	12.70					
47	35.0	12.29		3.76	1.12	0.93	+1.71
Calcium-rich diet.							
54	39.3	13.32	9.12	7.15	2.45	1.70	+3.00
61	35.4	12.71	9.00	2.94	1.54	1.97	−0.57
68	37.8	12.48	8.22	3.69	1.13	1.93	+0.63
75	38.6	12.62	9.00	4.17	1.33	2.24	+0.60
Rabbit 8 ♀, weight 3,710 gm.							
Control period.							
1	23.2	13.07					
11	35.4	12.21					
13	28.6	12.90					
17	30.8	11.10		4.69	1.29	1.40	+2.00
Calcium-rich diet.							
24	36.1	12.28	8.76	7.00	1.80	1.31	+3.89
31	27.1	12.76	9.65	3.16	1.07	2.50	−0.41
38	26.7	13.56	10.99	4.16	1.78	2.84	+0.54
45	25.5	12.43	10.20	5.27	1.94	3.14	+0.19

* Hematocrit values obtained by centrifuging in capillary tubes for 10 min. at approximately 3,600 revolutions per min.

† Calcium determinations in whole blood and plasma made by author's (15) method of direct precipitation.

‡ Calcium determinations in food, feces, and urine made by McCrudden's (9) method.

SUMMARY.

1. Intravenous or subcutaneous injections of calcium salts may cause a transitory increase in the calcium content of rabbit blood.

2. Feeding a calcium-rich diet has no effect on the calcium content of rabbit blood.

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TOXIC ACTION OF INGESTED LINSEED MEAL ON TROUT.*

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(From the Bureau of Fisheries, United States Department of Commerce, Washington.)

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Recently at a commercial hatchery in Rhode Island a peculiar disease broke out among brook trout of 1 and 2 years. The fish turned black and many of them became blind. Large numbers of them died. Just previous to death the affected fish would dart rapidly through the water, sometimes jumping entirely out of the water. After swimming nervously thus for a few seconds the fish would usually turn partly on the side and remain quiescent, as if dead, for an instant and would then resume its former unnaturally sluggish swimming. In most cases the fish would repeat this performance several times before finally succumbing. The superintendent was at a loss to explain the phenomenon but believed that the disease was connected in some way with the linseed meal, which formed part of a new food which the fish had been receiving for 6 or 7 weeks before the disease appeared. The same fish previously had been fed a mixture containing cottonseed meal.

Several weeks after linseed meal had been removed from the food, the affected fish, which had survived the disease, were for the most part still jet black in color and blind. They were thinner than normal fish, their blindness obviously handicapping them in their search for food. The vital organs appeared to be in normal condition. Microscopic examination of sections of the skin near the lateral line and just below the dorsal fin showed that the diameters of the melanophores had increased to nearly three times those of normal brook trout, thus accounting for the change in color of these fish.

* Published by permission of the Commissioner of Fisheries.

There is present in most linseed meals a glucoside which is usually partially split up, by enzymes present in the meal, into primary decomposition products, among which is hydrocyanic acid. According to Dunstan and Henry (1903–04) phaseolunatin, as the glucoside is called by them, is a dextrose ether of acetone cyanhydrin.

The non-toxicity of most linseed meals is due in large measure to the fact that decomposition of the glucoside does not take place in the stomach, because the enzyme has been destroyed by the high temperature to which the seed is subjected during the expression of the oil (Henry and Auld, 1908).

Cotton-seed meal is used extensively as a stock food. It has, however, a toxic action on pigs. Withers and Carruth (1913) have isolated from cotton-seed meal a substance called gossypol to which they have ascribed the toxic properties of the meal.

There are thus present in cotton-seed meal and in most unheated linseed meals substances which are toxic for certain animals. As both these meals were fed to the trout at the Rhode Island hatchery, the cotton-seed meal being fed from March to July and the linseed meal from July to the early part of September, it was thought advisable to include them both in a study of the causes of the disease, although what evidence had already been adduced seemed to prove that the linseed meal was solely responsible for causing the outbreak.

The observations and experiments recorded below were made at the commercial hatchery in Rhode Island and at the United States Government hatchery in White Sulphur Springs, West Virginia. The linseed meal employed in the entire study is the same as that which was in use at the commercial hatchery at the time of the outbreak of disease.

Experiments at the Rhode Island Hatchery.

The main object of the preliminary experiments was to determine which of the two meals had brought on the disease. It was considered of interest in this connection also to observe the effect of ingested raw linseed oil upon trout and to study the relative merits of cereal-meat meal mixtures and fresh hog lungs as foods for trout.

Accordingly, five lots of fish, each consisting of 300 yearling trout averaging about 5.2 inches in length and 0.97 ounce in weight, were placed in parallel ponds supplied with spring water of about 42° F. from a common trough at the head ends of the ponds. The fish of one lot (No. 1) were fed a mixture of Red Dog flour, meat meal, and water as a control. The fish of Nos. 2, 3, and 4 received feeds which were prepared by adding linseed meal, raw linseed oil, and cotton-seed meal, respectively, to the control ration. The fish of the remaining lot were fed ground hog lungs. The trout were given all the food they would take, being fed two or three and quite often four times a day.

TABLE I.

Composition of the Foods Received by the Trout at the Rhode Island Hatchery.

Lot No.	Food received.					
	Moisture.	Ash.	Ether extract (fat, etc.).	Protein.	Crude fiber.	Nitrogen-free extract (carbohydrate, etc.).
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	43.2	3.7	4.3	27.2	2.6	18.9
2	43.9	3.5	3.7	24.3	3.0	21.6
3	42.4	3.4	5.5	25.6	2.1	21.0
4	42.6	3.5	3.8	24.5	4.6	21.0
5	80.58	1.10	2.3	16.1		

The chemical composition of the five foods is given in Table I. The vegetable-meat meal mixtures were compounded in the proportions given in Table II and were obtained in the shape of short cylindrical particles of a doughy consistency by forcing them through a perforated plate. The particles were dusted with flour to prevent them from sticking together.

Results of Feeding Experiments.

The feces of all the fish receiving the cereal-meat meal mixtures were different from those of the fish receiving the lungs. The excrement of the latter was long, stringy, and voluminous, while that of the former was granular, like soaked rice in appearance, and comparatively small in volume.

At the end of 30 days of feeding no difference in the size, color, actions, or condition could be noted in the fish of Nos. 1, 3, 4, and 5. However, the fish of No. 2 at this time were in poor

TABLE II.

Results of Feeding Experiments Performed at the Rhode Island Hatchery.

Beginning of experiment, Jan. 27, 1918; end of experiment, Apr. 22.

Lot No.	Weight of 300 yearling brook trout	Average weight of fish.	Food.	Food consumed.	No. of dead fish.	Average weight of surviving fish.	Increase in weight.	Remarks.
		oz.		*lbs.*		*oz.*	*per cent*	
1	18 lbs. 4 oz.	0.97	16 parts Red Dog flour. 16 parts meat meal. 25 " water.	37.25	27	1.18	21.65	Appearance normal. Appetite good.
2	18 lbs. 2 oz.	0.97	11 parts Red Dog flour. 11 parts linseed meal. 11 parts meat meal. 25 " water.	Fair appetite during 1st week or 10 days only. Several turned black on Feb. 25. Food consumed to Mar. 8, 9.83 lbs. Dead fish to Mar. 8, 63. Many of the black fish appeared to have impaired vision.*				
3	18 lbs. 0.5 oz.	0.96	16 parts Red Dog flour. 16 parts meat meal 1 part linseed oil. 25 parts water.	39.67	63	1.26	31.25	Appearance fair. No black or blind fish.
4	18 lbs. 3 oz.	0.97	11 parts Red Dog flour. 11 parts cotton-seed meal. 11 parts meat meal. 25 " water.	41.33	12	1.20	23.71	Appearance normal. No black or blind fish.
5	16 lbs. 10 oz.	0.89	Fresh hog lungs.	116.5	2	1.55	74.16	Appearance excellent.

* On Mar. 21 the surviving fish of this lot were divided into two groups. The fish of one group (65 trout) were fed ground fresh beef spleen and the fish of the other group (81 trout) continued to receive the same food as previously. On Apr. 22 there remained 50 fish of the former group, including 2 black and blind fish and but 14 fish of the latter group.

condition; several had turned black but were not blind as yet. Their action was sluggish for the most part and they seemed to have difficulty in maintaining equilibrium. Occasionally a fish would begin a nervous twisting and darting movement through the entire length of the pond, exciting other fish to similar actions. At the completion of the spasm, the fish would come to rest and roll onto one side for a moment as if exhausted. Although quite a large number of the fish in this lot turned black during the entire course of the experiment, at no time were there more than three or four of them present simultaneously, the fish dying soon after turning black. The results of the feeding experiments carried on at the Rhode Island hatchery are recorded in Table II.

As a result of the above experiments, the following points are made clear: (1) of the two vegetable meals, linseed and cottonseed, the linseed meal alone is responsible for causing the pigment change, blindness, and death; (2) the causative agent is contained in the non-oil constituents of the linseed meal; (3) linseed oil in the food of trout has a slightly injurious effect upon the fish; (4) fish affected with linseed meal poisoning can be brought back to a healthy condition, except for the pigment change and blindness, by a diet of some fresh meat product; (5) yearling trout during 3 months on a diet of hog lungs gained in weight three times as fast as those receiving the cereal-meat meal mixtures; however, a three times greater gross weight of food was consumed in the former case than in the latter.

Experiments at the Hatchery at White Sulphur Springs, West Virginia.

When it had been determined that the principal disease manifestations were due to the non-oil constituents of linseed meal, it remained to be shown what part was played by the cyanogenetic glucoside. As previously stated, the glucoside occurring in linseed meal is not poisonous as such, but is injurious after hydrolysis of the hydrocyanic acid formed. The remaining feeding experiments were performed for the purpose of ascertaining, if possible, to what extent the disease symptoms brought on by the linseed meal were the result of the action of hydrocyanic acid.

The proportion of materials entering into the composition of the rations fed to the trout in these experiments is given in Table III. On the basis of an analysis of the linseed meal constitutent, Food B contained 0.0255 per cent of "free" hydrocyanic acid.[1] Foods E, F, G, and H were prepared by adding to the control ration (Food A) amounts of potassium cyanide which would bring their CN content equal to, one-fifth, triple, and five times that of Food B. Prior to its admixture with the other ingredients, the linseed meal of Food D was extracted with hot alcohol in a Soxhlet extractor for 24 hours to remove the glucoside and hydrocyanic acid (Auld 1911). Food C consisted of ground fresh hog lungs.

The manner of preparation of the foods containing vegetable and meat meals was the same as in the previous study. The small particles were thrown into the ponds in small amounts at a time and for the most part the fish devoured the food before it had an opportunity to sink to the bottom. There was little likelihood that the potassium cyanide in some of the foods was leeched out before the food was swallowed by the fish.

Results of Feeding Experiments.

A. Influence of the Water.

The results of the experiments are recorded in Table III. Attention should first be called to the unusual rise in the mortality of the fish in Nos. 6, 9, 10, and 11*a* from September 3rd to October 1st. If the fish of No. 6 had not been among those which had an increased mortality, one might well have assumed that the deaths in the other groups were due in large measure to the action of the hydrocyanic acid. The high death rate of the fish of No. 6 could not have been due solely to the food as this same ration of Red Dog flour and meat meal proved to be fairly satisfactory when fed to trout at the Rhode Island hatchery.

[1] Method for the analysis of "free" hydrocyanic acid in linseed meal: About 250 cc. of water are placed in a round bottomed, long neck flask of about 1.5 liters capacity, a few drops of toluene added, and the liquid is brought to the experimental temperature by immersion in a thermostat. A weighed quantity of the finely ground cake, 25 to 50 gm. according to circumstances, is then introduced into the flask, mixed by giving the contents a shake, and the flask then stoppered with a rubber bung, and placed in the thermostat. When the action has ceased (12 hours at 38°C.) the contents of the flask are steam-distilled into saturated sodium bicarbonate solution and the prussic acid content of the distillate is determined by titration with 0.02 N iodine solution. The production of a faintly yellow color, due to excess iodine, indicates the end of the reaction (Auld, 1911).

The water in the hatchery at White Sulphur Springs contains at times a marked excess of dissolved gases. At such times any fish in the fry stage are seriously affected unless measures are taken to readjust the air content of the water to more nearly a normal level by some means of aeration. Fingerling and adult fish hitherto have never been affected by it. It is possible that the increased death rate of these experimental fish was due to this one factor, but it is more plausible to believe that their death was due in part to this cause and in part to the effect of the prolonged feeding with the "artificial" foods, for the fish which were receiving hog lungs (Lots 7*a* and 12) at this time suffered but slight losses. The fish of No. 8 which received the cereal-meat meal mixture containing linseed meal that had been thoroughly extracted with hot 95 per cent alcohol to remove the glucoside did not show a high death rate until 2 weeks after the increased mortality began to take place among the fish in Nos. 6, 9, 10, and 11*a*. That the fish in No. 8 did not begin to die 2 weeks earlier was probably due to the fact that these fish were in better condition at this time than the other fish receiving cereal-meat meal mixtures because they had not been receiving the artificial food mixture long enough to affect them seriously.

B. Fresh Hog Lungs Compared with a Food Prepared from Dried Meals.

The fish of No. 6, fed on a diet of Red Dog flour and meat meal, during the first period suffered about the same from deaths as did the fish of No. 12 which received ground hog lungs. During the second period, however, the average number of fish dying per week in No. 6 was 8.2 while that in No. 12 was only 0.6. It is doubtful whether the mortality of the fish of No. 6 would have been so high during the second period if it had not been for the added effect of an unfavorable condition of the water in the ponds, that may have prevailed at this time. The fish of this lot were undoubtedly in poorer condition during the latter part of the experiment than were the fish of No. 12. The increase in weight of the fish of No. 6 and No. 12 during the experiment amounted to 116 per cent and 129 per cent, respectively, the difference in favor of the fish receiving hog lungs not

TABLE III.

Results of Feeding Experiments Performed at Hatchery at White Sulphur Springs, West Virginia.

Lot No.	Food.		Initial No. of fish.	Average initial weight of fish.	Deaths. 1st period, week ending July 16.	July 23.	July 30.	Aug. 6.	Aug. 13.	Average per week.	2nd period, week ending Aug. 20.	Aug. 27.	Sept. 3.	Sept 10.	Sept. 17.	Sept. 24.	Oct. 1.	Average per week.	Total.	Average weight of surviving fish.	Food consumed.
		oz.		*oz.*																*oz.*	*lbs.*
6	A	Red Dog flour, 8 Meat meal, 8 Water, 8	100	1.18	3	0	1	1	0	1	3	0	2	8	13	24	8	8.2	63	2.55	19.17
7	B	Red Dog flour, 5.3 Meat meal, 5.3 Linseed meal, 5.3 Water, 12	100	1.13	6	6	6	6	7	6.2									31	1.03	2.92
7*a* (Survivors of No. 7.)	C	Fresh hog lungs.	54								5	0	1	0	0	0	1	1	7	2.66	
8	D	Red Dog flour, 5.3 Meat meal, 5.3 Extracted linseed meal, 5.3 Water, 12	51	1.68							0	1	0	0	1	7	8	2.4	17	1.89	5.67

9	E	Red Dog flour, 8 Meat meal, 8 Water, 8 KCN, 0.0791 gm.	100	1.00	8	1	1	6	0	3.2	1	0	4	6	13	7	8	5.6	55	1.94	10.26
10	F	Red Dog flour, 8 Meat meal, 8 Water, 8 KCN, 0.0158 gm.	100	0.78	0	0	0	1	0	0.2	7	1	3	13	21	12	16	10.4	74	1.7	13.0
11	G	Red Dog flour, 8 Meat meal, 8 Water, 8 KCN, 0.2373 gm.	100	0.96	5	4	1	0	0	2.0									10	1.49	4.22
11*a* (Survivors of No. 11.)	H	Red Dog flour, 8 Meat meal, 8 Water, 8 KCN, 0.3955 gm.	66	1.49							0	2	1	6	9	14	8	5.7	40	1.92	7.35
12	C	Fresh hog lungs.	100	1.05	3	1	0	2	1	1.4	0	1	0	1	1	0	1	0.6	11	2.4	

being so marked as it was in the experiments performed at the Rhode Island hatchery. For some reason, possibly connected with the condition of the water and the somewhat apathetic attitude of the fish toward their food, the fish of No. 6 ate very little food during and after the week ending September 17th.

During the entire course of the experiment the fish of No. 6 appeared to be normal in health. No evidence of a pathological condition could be discerned. However, the ration which they received, consisting of equal weights of Red Dog flour, meat meal, and water, did not fulfill all the requirements of a good trout food. The fish receiving this ration, though they showed no apparent signs of ill health, suffered a somewhat higher mortality than the fish receiving the hog lungs. We must, therefore, conclude that, as a food for trout, such a mixture of flour and meat meal cannot be used to advantage to replace ground hog lungs or other fresh products of the packing house industry.

C. Effect of Ingested Linseed Meal on Trout.

During the entire 5 weeks of the feeding period from July 10th to August 13th, there was a weekly loss of about six fish in No. 7 receiving linseed meal (Food B). This loss was much greater than that which occurred in No. 9 which received an amount of potassium cyanide equivalent (molecularly) to the amount of hydrocyanic acid found by analysis in Food B. The greater toxicity of the linseed meal is probably ascribable to the action of linseed oil. It had earlier been determined that the linseed oil produces a slightly unfavorable influence on fish to which it is fed. It does not in itself cause the fish to turn black in color.

The fish of No. 7 began to act peculiarly within 8 days after the beginning of the feeding period. They darted hither and thither through the water occasionally. The nervousness of the fish was quite pronounced when they were disturbed by the sudden approach of someone to the edge of the pond. At such times some of the fish would jump out of the water in their excitement. They began to turn dark in color about 30 days after the experiment was started. Many of them at this time had sores on their snouts and fungus on their dorsal and adipose fins. Their appetite for Food B had fallen off also. After 35 days there

were two jet black fish among those surviving. Most of those which had died during the final days of the 5 weeks period were dark or black in color. The lower part of the intestines of nearly all the recently dead fish was very much inflamed. A like condition pertained in the living black fish.

The fish of No. 9 did not act peculiarly, that is, show signs of nervousness, until about 30 days after the feeding began. At this time they acted in a manner exactly similar to that of the fish of No. 7. There were no dark ones noted, however, until 40 to 45 days after the initial feeding, when many of the fish turned jet black in color. Furthermore, the lower part of the intestines of the affected fish was found to be congested with blood. After about 60 days the fish began to become infected with fungus.

The above observations would seem to prove that the pigment change and nervous condition were due primarily to the hydrocyanic acid of the linseed meal. The disease symptoms occurred much later in the fish of No. 9 than they did in those of No. 7. The belief that these symptoms were due entirely to the cyanide is strengthened by the fact that the fish of No. 6, receiving a ration identical in composition with that of Food E, except for the absence of potassium cyanide, did not give these manifestations. Furthermore, Food D, containing linseed meal from which the poisonous glucoside and the hydrocyanic acid had been removed by extraction with hot 95 per cent alcohol did not produce the pronounced "linseed meal" effect upon the fish of No. 8. The latter fish did become mildly nervous about 15 days after the initial feeding and were perhaps a little darker in color at the end of the experiment, though the color change was so slight as to make this observation of doubtful importance. The mortality of these latter fish was very low.

The fish of No. 7 at the end of 5 weeks were placed on a diet of ground hog lungs. The high mortality rate ceased almost at once and the fish somewhat regained their normal color. No totally blind fish were noted in this lot, as was observed in the case of the fish of No. 2 (Table II).

There is thus quite clear evidence that the outward manifestations of the disease, the pigment change and the excitability, produced in trout by the ingestion of linseed meal are due primarily to the hydrocyanic acid constituent of that food-stuff.

D. Effect upon Trout of Foods Containing Different Concentrations of Potassium Cyanide.

As previously stated, analysis showed that Food B contained 0.0255 per cent of "free" hydrocyanic acid, corresponding molecularly to 0.0614 per cent of potassium cyanide. If the figure 0.0614 is represented by a, then the proportions of potassium cyanide in Foods E, F, G, and H would correspond to a per cent, $\frac{a}{5}$ per cent, $3a$ per cent, and $5a$ per cent, respectively. Although during the first 7 or 8 weeks of the experiment the mortality of the fish receiving the rations containing potassium cyanide was a trifle higher than that of the control lot, the number of deaths during this time which might with certainty be attributed to the action of the cyanide is very small indeed. It happened that, during the latter half of the experiment, the greatest mortality occurred among the fish receiving the smallest amount of potassium cyanide (No. 10) and in this lot only were the number of deaths greater than in the control lot (No. 6). From these facts we are led to believe that the cyanide in the concentration and amount received by the trout had very little effect upon their mortality during the 12 weeks the fish were under observation.

The hydrocyanic acid of Foods E, F, G, and H did, however, produce disease symptoms in the fish. The food containing a per cent of potassium cyanide seemed to be more effective than the others in causing the fish to turn black and to get into an excitable state. Foods G and H, containing $3a$ per cent and $5a$ per cent of potassium cyanide, respectively, were a trifle less effective in this respect. Food F, containing $\frac{a}{5}$ per cent of potassium cyanide, was practically without any influence in this direction. It was noted that a number of the fish which had turned dark in color after having received for some time the foods containing either linseed meal or potassium cyanide were slightly defective in eyesight, quite a few being blind in one eye. It will also be recalled that a very large number of the black trout remaining in the preserves of the commercial hatchery after the management had stopped feeding linseed meal to the fish were totally blind.

The hydrocyanic acid present in the linseed meal, therefore, produces in trout partaking of a food consisting of equal parts by weight of Red Dog flour, meat meal, and linseed meal, a number of pronounced characteristic symptoms, among which are a nervous, excitable state, a change to a darker color or a jet black shade, and a weakened eyesight or blindness. However, the death of such fish appears to be due less to the action of the hydrocyanic acid than to some other factor. The possible cause for these effects will now be discussed.

Discussion of the Toxicity of Potassium Cyanide for Trout.

Pharmacologists state that prussic acid first stimulates then paralyzes the central nervous system in mammals. The lethal dose for man is about 0.05 to 0.08 gm. (Cushny, 1918). Assuming 145 or 150 pounds as the average weight of man, the lethal dose for man would be about 0.0003 to 0.0005 gm. per pound of body weight. The fish of No. 9 received an average of 1 gm. of food per fish per day for the first 21 days. They thus received an amount of potassium cyanide which corresponds molecularly to 0.00077 gm. of hydrocyanic acid per pound of fish per day. As the food was given to the fish twice daily and as some of the fish were more ravenous eaters than others, it is safe to say that many of these fish received as high as 0.0004 gm. of hydrocyanic acid, as potassium cyanide, per pound of body weight at one feeding. This proportion of acid to body weight in man, as above stated, is fatal. However, it did not appear to prove fatal to the fish. It caused many of them to turn black and blind and to act peculiarly. They seemed to be hypersensitive. Are all these symptoms related?

It is well known that certain fish possess the power of changing color to match the shade of their immediate background (Mast, 1914, and Bray, 1918). These colors are produced by concentration and dispersion of the pigment granules in the chromatophores of the skin. The movement of the granules is under the control of stimuli received through the eye, by way of the central and sympathetic nervous systems. When the eyesight of these fish is destroyed they can no longer simulate the background.

Blind trout are usually dark in color, often jet black. When a trout is blind in one eye, the opposite side will be black while the near side will be unchanged (Arens, 1904).

It appears that in trout as in other fish the stimuli received through the eyes are responsible for the adaptive changes in shade and then when vision is impaired the fish become dark in color or black. The amount of hydrocyanic acid received by the trout in these experiments, though not sufficient to kill the fish, apparently was large enough to produce a mild effect upon the central nervous system, causing the fish to become alternately hypersensitive and apathetic. It is the opinion of the authors that impaired vision caused the fish to turn dark in color. If the action of the acid was not too prolonged the fish resumed their natural color after a period of feeding on a cyanide-free ration.

SUMMARY.

1. Brook trout receiving a food mixture consisting of equal weights of Red Dog flour and meat meal did not gain in weight so fast as trout receiving fresh hog lungs, and the latter fish, after several weeks feeding, possessed a greater vitality or reserve strength than the former. Such a mixture is not an adequate substitute for the fresh, packing house products ordinarily used as food for trout.

2. Ingested raw linseed oil is slightly toxic to brook trout.

3. Ingested linseed meal produces in brook trout a nervous, excitable state, causes them to become black and blind, and proves fatal to a large proportion of the fish.

4. The injurious effect of ingested linseed meal upon brook trout is due in large measure to hydrocyanic acid which is present in most linseed meals as the result of the hydrolysis of a cyanogenetic glucoside. The amount of hydrocyanic acid ingested in the linseed meal at any given time was insufficient to produce death.

5. The nervousness, pigment change, and blindness are all believed to be referable to the action of the hydrocyanic acid on the nervous system of the trout. The observations reported herewith indicate that this action results in an impairment of the vision and a consequent interference with the control of the

movement of the pigment granules in the chromatophores of the skin, this control normally being obtained by means of stimuli received through the eyes by way of the central and sympathetic nervous systems.

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A STUDY OF THE ANTISCORBUTIC VALUE OF HONEY.

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The recent paper of Dutcher[1] showing that honey contains a small amount of antineuritic vitamine (probably due to included pollens rather than to the soluble constituents) naturally raises the question whether honey contains other vitamines as well. No investigation regarding antiscorbutic vitamine in honey has appeared and it was to throw light on this point that the present study was made.

A series of ten guinea pigs was fed on a diet of oats and water, which is known to produce scurvy in guinea pigs, and in addition white sage, comb honey bought in the open market and extracted from the comb on the day of administration was offered. In a preliminary period of from 5 to 6 days (Animals 1 to 6) it was found that undiluted honey was refused. A watery solution, one part of honey to fifteen parts of water, was accepted. This was administered for a period of 13 days. The strength was then increased to two parts of honey to fifteen parts of water. In the case of Animals 7 to 10 of the series, the 2:15 mixture was administered from the beginning up to the 21st day, when a 1:5 mixture was offered and taken. Daily inspection of the animals was made for tenderness of the joints, general behavior, and appearance of "face ache" position. The animals were weighed every 3 days. Though all the guinea pigs showed a progressive loss of weight, none, excepting No. 6, showed clinical evidence of scurvy until the 4th or 5th week. Marked tenderness of the knees was noted only in No. 6. This animal beginning on the 26th day behaved in a peculiar manner. When examined in the morning he was found lying on his back. When the knees were

[1] Dutcher, R. A., *J. Biol. Chem.*, 1918, xxxvi, 551.

TABLE I.

Effect on Guinea Pigs of Diet of Oats, Water, and Honey.

Animal No.	Initial weight.	Lived.	Final weight.	Died or killed.	Honey.		Scurvy.	Remarks.
					Total intake.	Average daily intake.		
	gm.	*days*	*gm.*		*cc.*	*cc.*		
1	525	42	300	Died.	190.4	6.03	++	Refused honey first 5 days. Honey intake measured last 30 days only. Teeth loose. Ribs: hemorrhage; ends enlarged; white line.* Hemorrhage of cecum and colon. Hemorrhage of right femur.
2	500	37	375	"	163.5	5.85	+++	Refused honey first 5 days. Honey intake measured last 28 days only. All teeth loose, cutting edges worn and black. Ribs: ends enlarged; white line; many hemorrhages. Large swellings and hemorrhages of both knees.
3	525	40	325	"	199.2	8.30	++	Refused honey first 5 days. Honey intake measured last 24 days only. Teeth loose. Ribs: ends enlarged; white line; several hemorrhages. Hemorrhage of one knee.
4	575	38	350	"	160.6	5.04	+	Refused honey first 6 days. Honey intake measured last 30 days only. Teeth slightly loose. Ribs: white line; 2 hemorrhages; no enlargements. Mandible much thinned.

* This "white line" is a macroscopic chalky white band of necrotic bone running transversely across the end of the rib at or near the cartilaginous junction, and probably corresponds with the radiographic "white line" found at the ends of the diaphyses of the long bones in human cases of scurvy. In guinea pigs with scurvy the costal white line is strikingly constant and is probably pathognomic of the disease.

TABLE I—*Concluded.*

Animal No.	Initial weight	Lived.	Final weight	Died or killed.	Honey.		Scurvy.	Remarks.
					Total intake.	Average daily intake.		
	gm.	*days*	*gm.*		*cc.*	*cc.*		
5	200	35	150	Died	137.9	5.31	+	Refused honey first 6 days. Honey intake measured last 26 days only. Teeth loose; alveolar hemorrhage. Ribs: few epiphyses enlarged. Hemorrhage in marrow of one femur.
6	200	27	175	Killed.	71.9	3.99	(?)	Refused honey first 6 days. Honey intake measured last 18 days only. Killed because of squealing when knees were pinched; weakness, etc. No gross lesions of scurvy. No microscopic examination.
7	200	26	130	Died.	214.4	8.25	++	Teeth loose. Ribs: ends enlarged; white line; several shafts fractured. Extensive hemorrhage of thoracic wall, left side. Hemorrhage of patellar ligament.
8	150	26	100	"	218.0	8.38	++	Teeth loose. Ribs: few small hemorrhages; ends enlarged; white line. Knees swollen and hemorrhagic.
9	210	26	150	"	161.4	6.21	++	Teeth loose. Ribs: several enlarged epiphyses; white line. Large recent hemorrhage of one knee; small hemorrhage of right knee. Subperiosteal hemorrhage of one femur.
10	150	30	125	"	191.8	6.39	+	Teeth not loose. Ribs: epiphyses enlarged; white line. Fresh hemorrhage of one knee.

pinched he squealed vigorously and when placed on his feet he moved about the cage in circles with an unsteady gait. The next morning he appeared to be normal but the following afternoon the peculiar symptoms reappeared. He was then killed but no characteristic gross lesions of scurvy were found. Through an unfortunate accident specimens for microscopic examination were not saved. It is quite possible that incipient scurvy, as suggested by the tenderness of the knees, was present. All the other animals died between the 27th and 42nd days and showed the characteristic lesions of scurvy, mostly of a recent and severe type.

The average daily intake of honey was between 3.99 and 8.30 cc. and between 0.88 and 5.58 cc. per 100 gm. of initial body weight. The degree of severity of the lesions was apparently unrelated to the amount of honey ingested.

It was at first thought because of the failure of scorbutic symptoms to develop until the 30th day in the six animals first tested that there was some evidence of protection against scurvy, since most animals fed on oats and water alone show signs of scurvy before this time.[2] The almost simultaneous appearance of severe scurvy in five of the six guinea pigs of the first set after the 30th day and in all of the last four at a still earlier period made such an assumption impossible. There seems to be no evidence that honey, at least in the quantities which guinea pigs can be induced to take, has any antiscorbutic value.

CONCLUSIONS.

1. In nine out of a series of ten guinea pigs fed on a diet of oats, water, and honey, severe scurvy developed in 4 to 6 weeks.

2. It is probable that no antiscorbutic vitamine is present in honey.

[2] Holst, A., and Frölich, T., *Z. Hyg.*, 1912, lxxii, 1.

SYNTHESIS OF PHYTIC ACID.

FIFTEENTH PAPER.

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INTRODUCTION.

Several attempts to synthesize phytic acid or inosite hexaphosphoric acid have been described in recent years. The methods have varied slightly but the principle in every experiment has been to obtain the desired product by heating together inosite and orthophosphoric acid.

Contardi[1] claimed to have synthesized phytic acid in this way and described his product as identical with the natural phytic acid isolated from plant material. Carré[2] was unable to substantiate the results of Contardi. Jegorow[3] using a similar method obtained a substance containing organic phosphorus. The writer[4] showed several years ago that inosite tetraphosphoric acid could be formed by heating inosite and phosphoric acid to 140–160°C. under reduced pressure.

Posternak[5] claimed recently to have synthesized phytic acid by heating a mixture of inosite, phosphoric acid, and phosphorus pentoxide. He states that the synthetic phytic acid was isolated as a crystalline double calcium-sodium salt which after drying at 120° had the composition $C_6H_{12}O_{27}P_6Ca_2Na_8$. The analytical data are not given, but a comparative study of the crystallography of the natural and the synthetic salts is described. Posternak calls this acid "inosite hexaphosphoric acid" but he represents it by the formula $C_6H_{24}O_{27}P_6$. This formula differs from

[1] Contardi, A., *Atti r. Accad. Lincei rendic.*, 1909, xviii, series 5, 64.
[2] Carré, P., *Bull. Soc. chim.*, 1911, ix, 195.
[3] Jegorow, M. A., *Biochem. Z.*, 1914, lxi, 41.
[4] Anderson, R. J., *J. Biol. Chem.*, 1912, xi, 471.
[5] Posternak, S., *Compt. rend. Acad.*, 1919, clxix, 138.

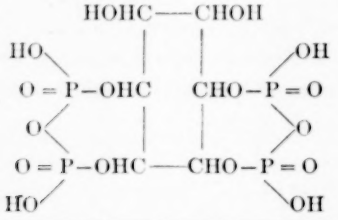
that of inosite hexaphosphoric acid, $C_6H_{18}O_{24}P_6$, by containing 3 molecules more of H_2O. How or in what manner these extra molecules of water are combined is not stated.

As a result of his earlier experiments to synthesize phytic acid by heating inosite and phosphoric acid, which only resulted in the formation of inosite tetraphosphoric acid; and in view of the fact that phytic acid always decomposes when heated into mixtures of inorganic phosphoric acid and lower inosite phosphoric acids, the writer was led to express the opinion[6] that phytic acid could never be synthesized by heating together inosite and phosphoric acid.

Since the method adopted by Posternak differed from those discussed above only by the introduction of phosphorus pentoxide, it appeared of interest to repeat his work. Accordingly the reaction between inosite and a mixture of phosphoric acid and phosphorus pentoxide has been studied. The conditions of the reaction as described by Posternak were followed closely.

From the reaction mixture was isolated an inosite phosphoric acid which was not identical in composition with the natural phytic acid although it had very nearly the same percentage of phosphorus. The substance differed not only in composition from phytic acid but its properties and reactions with reagents also differed from those of phytic acid in several particulars.

The composition of this new inosite phosphoric acid, as determined by the analyses of the barium and the silver salt, and of the free acid itself, agrees with the formula, $C_6H_{12}O_{16}P_4$. This corresponds to inosite dipyrophosphoric acid in which two hydroxyls of each molecule of the pyrophosphoric acid have reacted with two alcoholic hydroxyls of the inosite as indicated in the formula.



[6] Anderson, R. J., Thesis, Cornell University, 1919, 19.

It is not possible to state, however, whether the constitution of the acid is accurately indicated by this formula, for the inosite may possibly be combined with a more condensed phosphoric acid.

The barium salt of this new acid corresponds to the formula $C_{12}H_{18}O_{32}P_8Ba_3$. Such a substance is either a mixture of the mono- and dibarium salts or else 2 molecules of the monobarium salt are united by 1 atom of barium.

$$C_6H_9O_{16}P_4Ba$$
$$\diagdown\!\!\diagup Ba$$
$$C_6H_9O_{16}P_4Ba$$

The silver salt is precipitated from a slightly acid solution, on addition of silver nitrate, as a white amorphous precipitate which corresponds to the tetrasilver salt, $C_6H_8O_{16}P_4Ag_4$.

EXPERIMENTAL.

First Synthesis.

Dry orthophosphoric acid, 56 gm., was heated in a flask to 100°, and 12 gm. of dry inosite were added. The inosite was dissolved by continuing the heat between 100–110°. To this solution were added 90 gm. of phosphorus pentoxide in small portions. A very considerable increase in the temperature was observed when the phosphorus pentoxide was added. The mixture was well stirred by shaking and, finally, by a glass spatula. The flask containing the reaction mixture was then heated in an oil bath to 120–130° for 3 hours. It was cooled to room temperature, about 200 cc. of water were added, and the mixture of acids was dissolved by adding a 20 per cent solution of sodium hydroxide until the reaction was faintly alkaline to litmus. The solution was then filtered and concentrated on the water bath and set aside to allow sodium phosphate, pyrophosphate, etc., to crystallize.

The crystals were filtered off on a Buchner funnel and washed with several small portions of cold water. The filtrate was made up to 500 cc. with water, 25 gm. of sulfuric acid were added, and the solution was heated to 100° for 1 hour, for the purpose of

converting any pyrophosphoric acid into orthophosphoric acid. It has been shown by Plimmer[7] that phytin is not appreciably hydrolyzed by such treatment. Sodium hydroxide was then added until the reaction was only faintly acid.

The organic and the inorganic phosphoric acids which were present in the solution were then precipitated by adding a concentrated solution of copper acetate in excess. The copper precipitate was filtered on a Buchner funnel and washed until free from sulfates; then it was suspended in water and decomposed with hydrogen sulfide. The copper sulfide was filtered off and the excess of hydrogen sulfide removed by a current of air. To the filtrate, about 5 liters, were added 400 gm. of barium chloride, resulting in a heavy white amorphous or granular precipitate. This precipitate was filtered and washed with water until free from chlorides. The addition of an equal volume of alcohol to the filtrate produced a voluminous white precipitate which was purified as will be described later.

Purification of the Substance Precipitated by Barium Chloride.

The moist precipitate was rubbed up to a thin paste with water and brought into solution by adding dilute hydrochloric acid drop by drop. It was then filtered and precipitated by adding a solution of barium hydroxide. The barium salt was filtered and washed free of chlorides with water and again dissolved in dilute hydrochloric acid, filtered, and precipitated by adding an equal volume of 95 per cent alcohol. The above mentioned alternate precipitations with barium hydroxide and alcohol from dilute hydrochloric acid were repeated four times. The substance was further precipitated twice with alcohol from dilute hydrochloric acid, finally washed with dilute alcohol and alcohol and ether, and dried in vacuum over sulfuric acid. It was a snow-white amorphous powder and weighed 7.3 gm.

Various attempts were made to crystallize this barium salt by the method formerly used in purifying barium phytate,[8] but without success. The amorphous salt was free from chlorides and inorganic phosphate and it did not contain any weighable

[7] Plimmer, R. H. A., *Biochem. J.*, 1913, vii, 72.

[8] Anderson, R. J., *J. Biol. Chem.*, 1914, xvii, 141, 151, 165, 171.

quantity of sodium. When dissolved in dilute nitric acid it gave a pure white flocculent precipitate with ammonium molybdate. In strong solutions this precipitate appears immediately while in very dilute solutions it comes down only on warming. The precipitate remains pure white in color and does not turn yellow on warming to 65° or on standing at room temperature for a long time. Before the inorganic phosphate had been removed by the repeated precipitation of the barium salt by alcohol from dilute hydrochloric acid, the white precipitate produced by the ammonium molybdate assumed a yellow color either immediately or very gradually, depending upon the amount of inorganic phosphate which was present.

The substance was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.2980 gm. substance gave 0.0427 gm. H_2O and 0.1121 gm. CO_2.
0.1577 " " " 0.0861 gm. $BaSO_4$ and 0.1018 gm. $Mg_2P_2O_7$.
Found: C = 10.26; H = 1.60; P = 17.99; Ba = 32.12 per cent.
For $C_{12}H_{18}O_{22}P_8Ba_3$ = 1,334, calculated: C = 10.79; H = 1.34; P = 18.59; Ba = 30.88 per cent.

Deducting the amount of barium found and allowing for its equivalent as hydrogen and water the composition of the free acid was calculated as follows.

C = 15.01; H = 3.03; P = 26.34 per cent.
For $C_6H_{12}O_{16}P_4$ = 464, calculated: C = 15.51; H = 2.58; P = 26.72 per cent.

Examination of the Substance Precipitated by Alcohol.

The white amorphous precipitate obtained by the addition of alcohol to the filtrate from the above barium salt was purified by precipitating it alternately fifteen times with barium hydroxide and alcohol from dilute hydrochloric acid in the manner described above. The snow-white amorphous product finally obtained gave in nitric acid solution a voluminous white precipitate with ammonium molybdate which gradually turned yellow on warming, thus showing the presence of inorganic phosphate. The substance was then dissolved in 200 cc. of very dilute hydrochloric acid and precipitated by adding 200 cc. of a saturated

solution of barium chloride. The heavy white precipitate which separated was filtered, washed, and again precipitated several times from dilute hydrochloric acid by alcohol. After finally filtering, the substance was washed free from chlorides with dilute alcohol and then washed in alcohol and ether and dried in vacuum over sulfuric acid. The snow-white product weighed 4 gm. Dissolved in dilute nitric acid it gave a voluminous pure white precipitate with ammonium molybdate which did not turn yellow on heating. The inorganic phosphate had, therefore, been removed completely.

The substance was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.3343 gm. substance gave 0.0399 gm. H_2O and 0.1031 gm. CO_2.
0.1768 " " " 0.1058 gm. $BaSO_4$ and 0.1135 gm. $Mg_2P_2O_7$.
Found: C = 8.41; H = 1.33; P = 17.89; Ba = 35.21 per cent.
Calculated to free acid: C = 12.88; H = 2.83; P = 27.41 per cent.

The composition differs considerably from that of the first preparation obtained by precipitating with barium chloride, the percentage of carbon being lower and that of phosphorus higher. The composition approaches that of phytin or inosite hexaphosphoric acid, but its properties differ from those of phytin in that it is precipitated from dilute hydrochloric acid solutions by barium chloride and, also, in that its solution in dilute nitric acid gives a white amorphous precipitate with ammonium molybdate. Further, it was not possible to obtain any crystalline barium salts by the method used with barium phytates. Another difference in behavior was noticed in that the addition of water to the dilute hydrochloric acid solution of the barium salt gave a white precipitate.

This substance was probably a mixture of inosite phosphoric acids, but the nature of these esters could not be determined.

Second Synthesis.

Since in the first experiment no evidence had been obtained of the formation of phytic acid or inosite hexaphosphoric acid, it was decided to study the reaction between inosite, orthophosphoric acid, and phosphorus pentoxide under conditions varying

from that described above. A second synthesis was therefore carried out as follows:

Inosite, 9 gm., dry phosphoric acid, 39 gm., and phosphorus pentoxide, 39 gm., were mixed and heated in a flask exactly as described before except that the temperature of the oil bath did not exceed 120° and the mixture was heated for only 1 hour. The reaction mixture was worked up exactly as previously described and the substance precipitated by barium chloride was purified in the same manner as the first preparation by precipitating alternately with barium hydroxide and alcohol from dilute hydrochloric acid until the dilute nitric acid solution gave a pure white precipitate with ammonium molybdate. The product thus prepared was a snow-white powder which weighed 6.8 gm. after drying in vacuum over sulfuric acid. It was free from chlorides and inorganic phosphate and did not contain any bases other than barium.

The substance was analyzed after drying at 110° in vacuum over phosphorus pentoxide.

0.3993 gm. substance gave 0.0558 gm. H_2O and 0.1548 gm. CO_2.
0.1835 " " " 0.0938 gm. $BaSO_4$ and 0.1220 gm. $Mg_2P_2O_7$.
Found: C = 10.57; H = 1.56; P = 18.53; Ba = 30.08 per cent.
Calculated to free acid: C = 15.03; H = 2.83; P = 26.34 per cent.

Judging by the analytical result this product is evidently identical with the one obtained in the first synthesis.

Third Synthesis.

In a third experiment 9 gm. of inosite, 39 gm. of dry phosphoric acid, and 39 gm. of phosphorus pentoxide were heated for 1 hour in an oil bath having a temperature of 115–120°. The reaction product was isolated in the same manner as before. The substance after drying in vacuum over sulfuric acid weighed 11.7 gm. It was free from chlorides and inorganic phosphate, and bases other than barium were absent.

For analysis the substance was dried in vacuum at 105° over phosphorus pentoxide.

0.3543 gm. substance gave 0.0535 gm. H_2O and 0.1419 gm. CO_2.
0.1891 " " " 0.0913 gm. $BaSO_4$ and 0.1270 gm. $Mg_2P_2O_7$.
Found: C = 10.92; H = 1.68; P = 18.72; Ba = 28.41 per cent.
Calculated to free acid: C = 15.17; H = 2.90; P = 25.99 per cent.

The percentage of barium varied in these different preparations yet when the barium is deducted, allowing for corresponding quantities of hydrogen and water, fairly concordant results are obtained for the composition of the acid. These analytical results as shown below agree with an acid having the composition $C_6H_{12}O_{16}P_4$.

For $C_6H_{12}O_{16}P_4$ calculated . . C = 15.51; H = 2.58; P = 26.72 per cent.
Found first synthesis . . . . . C = 15.01; H = 3.03; P = 26.34 " "
" second " . . . . . C = 15.03; H = 2.83; P = 26.34 " "
" third " . . . . . C = 15.17; H = 2.90; P = 25.99 " "

Purification of the Barium Salt.

In the hope of obtaining a homogeneous crystalline salt of this acid the following experiment was made. The three barium precipitates of identical composition obtained by precipitating with barium chloride were united. The mixture weighed 22.8 gm. It was rubbed up to a fine thin paste with 200 cc. of water and brought into solution by adding just sufficient dilute hydrochloric acid. The solution was filtered and to it was added slowly and with constant shaking a concentrated solution of 11 gm. of barium chloride. The precipitate which formed at first dissolved on shaking and warming the mixture to about 65°. After adding all the barium chloride a slight permanent cloudiness remained which did not clear up on warming. The solution was then allowed to cool and to stand at room temperature over night. A heavy white powder separated gradually. This substance was not crystalline but it consisted, as shown under the microscope, of uniform transparent granules or globular particles.

This substance was filtered off, washed with water and then with alcohol and ether, and allowed to dry in the air. The snow-white powder weighed 3.1 gm. In nitric acid solution it gave no reaction with silver nitrate, and ammonium molybdate produced a voluminous pure white precipitate indicating that it was free from chlorides and inorganic phosphate. To the filtrate from

this preparation was added a concentrated solution of 20 gm. of barium chloride in the same manner as before until a faint permanent cloudiness remained. After standing over night a further quantity of the globular precipitate had separated which, under the microscope, appeared to be identical with the first preparation. This was filtered off, washed and dried as before, and was also found to be free from chlorides and inorganic phosphate.

These preparations were analyzed after drying to constant weight at 105° in vacuum over phosphorus pentoxide.

The first globular preparation gave the following results on analysis.

0.3784 gm. substance lost 0.0536 gm. on drying.
0.1813 " " " 0.0257 " " "
0.3248 " " gave 0.0463 gm. H_2O and 0.1267 gm. CO_2.
0.1476 " " " 0.0757 gm. $BaSO_4$ and 0.0986 gm. $Mg_2P_2O_7$.
Found: C = 10.63; H = 1.59; P = 18.62; Ba = 30.18; H_2O = 14.16 per cent.
For $C_{12}H_{18}O_{32}P_8Ba_3 = 1,334$, calculated: C = 10.79; H = 1.34; P = 18.59; Ba = 30.88 per cent.
For 12 H_2O, calculated: 13.93 per cent.

The second preparation gave the following results.

0.2834 gm. substance gave 0.0382 gm. H_2O and 0.1050 gm. CO_2.
0.1887 " " " 0.1062 gm. $BaSO_4$ and 0.1224 gm. $Mg_2P_2O_7$.
On drying it lost 14.61 and 14.51 per cent of water.
Found: C = 10.10; H = 1.50; P = 18.08; Ba = 33.12 per cent.

In these preparations the percentage of barium again varies, but on deducting the amount of barium found and allowing for a corresponding quantity of hydrogen and water the following results are obtained.

First globular precipitate. . . C = 15.14; H = 2.88; P = 26.30 per cent.
Second " " . . . C = 15.01 H = 2.92; P = 26.84 " "
Calculated for $C_6H_{12}O_{16}P_4$. C = 15.51; H = 2.58; P = 26.72 " "

Preparation of the Acid.

The free acid was prepared from 3 gm. of the globular salt. The barium was removed with a slight excess of sulfuric acid and the barium sulfate filtered off. The filtrate was precipitated with an excess of copper acetate, filtered, and washed free of sul-

fates. The copper salt was decomposed in aqueous suspension by hydrogen sulfide. The copper sulfide formed an exceedingly persistent colloidal solution which it was very difficult to break up and so much time was consumed in this operation that some hydrolysis of the acid occurred, as shown by the fact that its dilute solution gave a white precipitate with ammonium molybdate which gradually turned yellow in color. Equally poor success was experienced with a second preparation of the free acid in which lead was substituted for copper. After the copper sulfide had finally been removed the solution was concentrated under reduced pressure at a temperature not exceeding 40° and then dried in vacuum over sulfuric acid. The acid was obtained as a colorless thick syrup which quickly dried forming a hard brittle mass. It was readily soluble in water and alcohol and it showed no tendency whatever to crystallize. The addition of ether to the alcoholic solution of the acid caused it to separate as small oily drops.

The acid was analyzed after drying to constant weight in vacuum at 78° over phosphorus pentoxide. On drying at this temperature the acid darkened slightly in color indicating some decomposition.

0.1464 gm. substance gave 0.0379 gm. H_2O and 0.0813 gm. CO_2.
0.1588 " " " 0.1480 gm. $Mg_2P_2O_7$.
Found: C = 15.15; H = 2.89; P = 25.97 per cent.
0.1565 gm. of the dry acid dissolved in 50 cc. of water required 15.4 cc. of 0.1 N NaOH using phenolphthalein as indicator.
For $C_6H_8O_3\,[P_2O_3(OH)_2]_2$, calculated: 13.5 cc. of 0.1 N NaOH.

After titrating the above, an excess of neutral barium chloride was added which caused a white precipitate of the barium salt and at the same time an increase in the acidity which required 5.9 cc. of 0.1 N NaOH for neutralization. If we assume that the two free alcoholic hydroxyls in the inosite ring became acidic by reason of the presence of the adjacent acid molecules a hexa-basic acid would then be formed. For $C_6H_6(OH)_2O_4\,[P_2O_3(OH)_2]_2$, calculated: 20.2 cc. of 0.1 N NaOH. As will be noticed, an excess of alkali was required but this was probably due to the slight hydrolysis of the acid during the preparation, of which mention has been made.

Properties of the Free Acid.

The aqueous solution of the acid gave white amorphous precipitates on adding silver nitrate, barium chloride, or calcium chloride. Magnesium chloride gave no precipitate in the cold but on warming a white precipitate was formed which redissolved on cooling. Copper sulfate in excess gave a bluish white precipitate. Ferric chloride gave a yellowish white precipitate which was very insoluble in hydrochloric acid. A dilute solution of the acid immediately precipitated egg albumin. As stated before ammonium molybdate produced, even in dilute solutions, a white flocculent precipitate either immediately or on warming; this precipitate, owing apparently to partial hydrolysis of the acid during its preparation, gradually turned yellow in color when the solution was warmed.

Phytic acid is not precipitated from aqueous solutions by barium or calcium chlorides. Ammonium molybdate produces a white granular or crystalline precipitate with phytic acid only in concentrated solution.

Preparation of the Silver Salt.

An aqueous solution of the acid was nearly neutralized with ammonia and then acidified by adding a few drops of nitric acid. Silver nitrate was added in slight excess and the white amorphous precipitate was filtered, washed in water and alcohol and ether, and dried in vacuum over sulfuric acid. The resulting silver salt was a nearly white amorphous substance which was very slightly sensitive to light and on moist litmus paper it showed only a faint acid reaction.

The salt was analyzed after drying at 105° in vacuum over phosphorus pentoxide.

0.2584 gm. substance gave 0.0257 gm. H_2O and 0.0773 gm. CO_2.
0.2359 " " " 0.1497 gm. AgCl and 0.1156 gm. $Mg_2P_2O_7$.
Found: C = 8.15; H = 1.11; P = 13.66; Ag = 47.76 per cent.
For $C_6H_3O_{15}P_4Ag_4$, calculated: C = 8.07; H = 0.89; P = 13.91; Ag = 48.40 per cent.

Hydrolysis of the Synthetic Preparation into Inosite and Phosphoric Acid.

The barium salt was hydrolyzed by heating in an autoclave to 140–150° for 2½ hours with dilute sulfuric acid. After cooling, the inosite was isolated in the usual way and recrystallized several times from water with the addition of alcohol. The quantity of pure recrystallized inosite which was obtained corresponded to 84 per cent of the theoretical amount. The substance gave the reaction of Scherer and melted at 223° (uncorrected). That it was pure inosite was shown by its crystal form, melting point, and the Scherer reaction, and analysis was therefore omitted.

SUMMARY.

A study has been made of the reaction between inosite, phosphoric acid, and phosphorus pentoxide.

The conclusion of Posternak that the organic phosphoric acid produced in this reaction is identical with the natural phytic acid or inosite hexaphosphoric acid, $C_6H_{18}O_{24}P_6$, or as formulated by Posternak, $C_6H_{24}O_{27}P_6$, could not be confirmed.

The only product which could be isolated in approximate purity from the reaction mixture corresponded to an inosite ester of pyrophosphoric acid containing 4 atoms of phosphorus or 2 molecules of pyrophosphoric acid. Traces of other inosite phosphoric acids are undoubtedly formed but the above substance represents the principal product of the reaction.

This new acid corresponds to the formula, $C_6H_{12}O_{16}P_4$. It resembles phytic acid in that it contains very nearly the same percentage of phosphorus but its properties and reactions differ in several important particulars from those of phytic acid.

The synthesis of phytic acid or inosite hexaphosphoric acid cannot be considered as accomplished and it appears doubtful if this substance can be successfully synthesized by the methods heretofore employed.

EXPERIMENTS ON CARBOHYDRATE METABOLISM AND DIABETES.

II. THE RENAL THRESHOLD FOR SUGAR AND SOME FACTORS MODIFYING IT.

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Attention was called in the preceding communication[1] to the importance of renal permeability in determining the apparent tolerance for sugar. The present group of papers, together with that of Palmer[2] on tissue permeability, are fragments of a research originally planned on the passage of sugar through different body membranes in diabetic and non-diabetic conditions. Publication of the following incomplete observations is ventured because of the apparent suggestiveness of some of them.

No detailed survey of the literature on the permeability of the kidney for sugar will be attempted. Some of the older literature was reviewed by Allen,[3] and the principal facts bearing on the present topic may be summarized as follows: (*a*) a brief glycosuria is attended by a lowering of the sugar threshold of the normal kidney, so that as the hyperglycemia is subsiding sugar continues to pass into the urine with a lower level of blood sugar than that which is necessary to cause glycosuria at the outset; (*b*) prolonged glycosuria or hyperglycemia is attended with an elevation of the sugar threshold, so that glycosuria may remain absent with a blood sugar level considerably higher than that at which sugar excretion ordinarily occurs; (*c*) renal abnormalities, either spontaneous diseases or drugs and other agencies, may either increase

[1] Allen, F. M., and Wishart, M. B., *J. Biol. Chem.*, 1920, xlii, 415.

[2] Palmer, W. W., *J. Biol. Chem.*, 1917, xxx, 79.

[3] Allen, F. M., Studies concerning glycosuria and diabetes, Cambridge, 1913, 44, 384 ff., 541.

or diminish the permeability. Epstein and Baehr[4] and Woodyatt[5] have emphasized the importance of blood volume in addition to the percentage concentration of sugar for governing the excretion. This, however, is presumably only one of many factors influencing renal permeability. The present observations were accompanied with hemoglobin and corpuscle percentage determinations, as illustrated in the following and other papers, and were not explainable by blood volume changes as far as could be judged by these methods.

Benedict and Osterberg[6] have recently placed this subject on a new basis, by devising a quantitative method for the sugar in normal urine. With this procedure there is no renal threshold for glucose, and various metabolic conditions can be more accurately studied in their influence upon the kind as well as the quantity of urinary carbohydrate. Two excuses may be offered for presenting observations based upon the older idea of the renal threshold and a fixed distinction between glycosuria and its absence as opposed to the newer concept of normal and pathological variations of glycuresis. One is that so many matters of experimental and clinical importance have been connected with the excretion of sugar according to the older standard, that though this may now be subject to modification it can scarcely lose its significance altogether. The other is the likelihood that all valid standards will be found to agree, and that the finer observations with the new method may in general confirm the cruder ones with the old. The present findings may therefore at least suggest causes of altered renal permeability which are worth investigating by the improved method.

A large number of parallel analyses of urine by copper reduction according to Benedict and of blood by the Benedict picric acid method have been performed in the course of the entire investigation, but relatively few are suitable for fixing the threshold or permeability. Consideration is therefore limited chiefly to forty-nine dogs, from which parallel samples of blood and urine were obtained at 15 minute or other suitable intervals, or the

[4] Epstein, A. A., and Baehr, G., *J. Biol. Chem.*, 1914, xviii, 21. Epstein, A. A., *Proc. Soc. Exp. Biol. and Med.*, 1915–16, xiii, 67; *Am. J. Med. Sc.*, 1917, cliv, 103.

[5] Woodyatt, R. T., *The Harvey Lectures*, 1915–16, xi, 326.

[6] Benedict, S. R., and Osterberg, E., *J. Biol. Chem.*, 1918, xxxiv, 195.

urine alone was collected frequently and a blood sample taken as soon as a reducing reaction appeared or disappeared. The experiments were seldom performed directly for this purpose, but by bearing this point in mind it was easily included as an incidental observation in experiments of various kinds. The number of observations is multiplied by the fact that they were repeated in all the above dogs under different experimental conditions. The animals most often mentioned are those in which the changes were followed through successive stages of diabetes. The results are briefly summarized under the respective types of experiments (fasting, different injections, feedings, etc.), and the individual dogs under each heading are arranged in order according to the gradations of tolerance or diabetes.

The degree of diabetes is naturally something which cannot be defined exactly, but for this purpose dogs were considered severely diabetic if they showed glycosuria on protein diet, and mildly diabetic if considerable quantities of carbohydrate were necessary for glycosuria. Such diabetes was merely "potential" except on test days, because active symptoms were kept absent as a rule by appropriate diets. Other dogs had undergone removal of $\frac{3}{4}$ or more of the pancreas, but not enough to produce diabetes. These and the normal dogs were on bread and soup diets. The others were on such protein diets as were necessary for sugar freedom, with addition of fat sometimes as specified.

Fasting Plasma Sugars.

The dogs were fed between 10 a.m. and noon, and the blood samples designated as "fasting" were taken before feeding the next day. Table I gives a summary of the results.

As the conditions specified preclude any glycosuria, this group naturally includes no observations of threshold. The plasma sugar values found for the normal animals agree with those in the literature and with numerous others in the course of this research. Those of the partially depancreatized non-diabetic animals are entirely similar, there being no tendency to fasting hyperglycemia from the removal of pancreas tissue to any point short of diabetes. They may, however, be more readily subject to hyperglycemia from slight excitement or other disturbance,

thus accounting for the maximum of 0.154 per cent. The values are observed to rise with increasing severity of diabetes. The important point in the present connection is that these levels were reached and maintained for long periods without glycosuria. As heavy glycosuria always accompanies such values normally, it must be concluded that there is a marked elevation of the sugar threshold with increasing severity of diabetes. This corresponds to the observations in the great majority of human cases.

The rôle of the long continued excess of sugar itself will be discussed below. Some part may be attributed to it in most of the above animals, as also in most human cases, because of the history of long continued glycosuria or hyperglycemia in most of

TABLE I.

Condition.	No. of dogs.	No. of analyses.	Plasma sugar.		
			Maximum.	Minimum.	Average.
			per cent	*per cent*	*per cent*
Normal	10	14	0.121	0.070	0.108
Partially depancreatized non-diabetic	10	46	0.154	0.065	0.109
Mild diabetes	11	43	0.200	0.084	0.143
Severe diabetes	18	51	0.357	0.109	0.196

them. That a very prolonged excess of sugar, however, is not essential to such elevation of the threshold is proved by the records of certain animals.

Dog D4-28 was previously described.[7] $\frac{1}{4}$ of the pancreas was removed[8] on Sept. 28, 1916, and the dog was radically undernourished, so that hyperglycemia was brief and rare. Nevertheless in a feeding test on Dec. 1, 1917, the plasma sugar went to 0.216 per cent without glycosuria. It may be worth noticing that the diet had contained considerable fat.

Dog F6-08, a male Dalmatian aged 3 years, weighing 15 kilos, on Dec. 6, 1917, underwent removal of 27.9 gm. of pancreatic tissue, leaving a remnant estimated at 1.4 gm. ($\frac{1}{21}$). With fasting there was no glycosuria, but the plasma sugar on the morning of Dec. 8 was 0.179 per cent. Additional bits of pancreatic tissue were removed for microscopic examination on Dec. 14 and 19. 100 gm. of bacon grease were fed daily after Dec. 24, no other food being given. Dec. 31, at a weight of 8.5 kilos, the plasma sugar was 0.256 per cent without glycosuria.

[7] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 581–583.

[8] All operations were performed under ether anesthesia.

Dog F6-09,a shaggy male mongrel aged 8 years, weighing 18.75 kilos, on Dec. 6, 1917, underwent removal of 36.2 gm. of pancreatic tissue, leaving a remnant estimated at 2.6 gm. ($\frac{1}{7}$). With fasting there was no glycosuria, and the plasma sugar was 0.242 per cent on Dec. 8 and 0.192 per cent on Dec. 10. Without food and without glycosuria, the plasma sugar had risen by Dec. 13 to 0.313 per cent.

Dog F6-11, a female mongrel aged 6 years, weighing 19 kilos, on Dec. 19, 1917, underwent removal of pancreatic tissue weighing 26.3 gm., leaving a remnant estimated at 1.9 gm. ($\frac{1}{14}$ to $\frac{1}{5}$). With fasting and continuous absence of glycosuria, the plasma sugar on Dec. 26 was 0.278 per cent and on Dec. 27, 0.357 per cent.

Subcutaneous Glucose Injections.

Glucose was injected subcutaneously in fasting dogs in 30 per cent solution. Satisfactory threshold figures were obtained in only three partially depancreatized non-diabetic animals and one with mild diabetes.

Dog B2-00, weighing 14 kilos (at a stage when removal of about 1 gm. of additional pancreatic tissue was found necessary to produce diabetes), had a fasting plasma sugar of 0.065 per cent, and received 3 gm. of glucose per kilo subcutaneously. Slight glycosuria occurred with 0.163 per cent of plasma sugar, and a trace could still be detected when the plasma sugar had fallen to 0.118 per cent.

Dog B2-01, with a similar weight and condition, had a fasting plasma sugar of 0.099 per cent, and received 4 gm. of glucose per kilo subcutaneously. With the first trace of glycosuria the plasma sugar was found to be 0.151 per cent. 5 months later the same dose was given. Starting with a fasting plasma sugar of 0.106 per cent, with the first trace of glycosuria the plasma sugar was found to be 0.147 per cent. The threshold had thus apparently not changed with time in a non-diabetic dog on bread diet.

Dog B2-61, weighing 5 kilos and possessing $\frac{1}{4}$ of the pancreas, received 3 gm. of glucose per kilo and showed almost a titratable glycosuria when the plasma sugar reached 0.147 per cent (from a fasting level of 0.106 per cent).

Dog B2-79, weighing 15 kilos, in the stage of mild diabetes, with a remnant of $\frac{1}{4}$ of the pancreas, was kept nearly constantly free from glycosuria on a diet of beef lung, and 5 months after operation received 5 gm. of glucose per kilo subcutaneously. Starting with fasting plasma sugar of 0.105 per cent, a strong trace of sugar appeared in the urine with a plasma sugar of 0.182 per cent. The threshold thus seemed slightly elevated as compared with the non-diabetic animals.

Intravenous Glucose Injections.

With the method of discontinuous injections described in the preceding paper,[1] the threshold of disappearance of glycosuria after the injections are finished is all that can be determined accurately.

Three normal dogs were thus observed, which received 1.5 gm. of glucose per kilo per hour. In one of these glycosuria disappeared while the plasma sugar fell from 0.173 to 0.150 per cent, and in another while the plasma sugar fell from 0.154 to 0.143 per cent. In the third there was a very faint urinary reaction at 0.167 per cent of plasma sugar, and with completely negative urine the plasma sugar was down to 0.128 per cent.

Dog B2-00, partially depancreatized just short of diabetes, received similar dosage and became aglycosuric as the plasma sugar fell from 0.145 to 0.130 per cent.

Dog B2-01, with very mild diabetes, showed a very low threshold as noted in the preceding paper,[9] the urine giving a slight copper reduction during this time while the successive plasma sugars were 0.084, 0.087, and 0.116 per cent.

Three other dogs with slightly greater diabetic tendency showed thresholds between 0.140 and 0.123 per cent, between 0.228 and 0.200 per cent, and between 0.212 and 0.200 per cent, respectively. Here the first dog with the lowest threshold had the smallest pancreas remnant but had also been kept freest from glycosuria and hyperglycemia.

The concentration of the solutions used in the above mentioned tests ranged from 5 to 20 per cent, but showed no constant relation to the different thresholds of disappearance of glycosuria. On the other hand, comparisons may be made with identical doses at different stages of diabetic progress in a dog previously described.[10] Here the thresholds are not exact, because the hour intervals are too long for accuracy, but the comparative relation holds. The table in the reference cited shows that on August 6, 1917 (3 days after the final pancreas operation which produced very mild diabetes), during 2 hours following the last injection there were traces of glycosuria with plasma sugars of 0.125, 0.128,

[9] Allen and Wishart,[1] p. 439.

[10] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 567.

and 0.130 per cent, successively. Glycosuria was then absent in the next hour during which the plasma sugar rose to 0.133 per cent (indicating disappearance of the special glycosuric tendency which persists for a time during the decline of a glycosuria). On November 19, 1917, there was only a faint trace of glycosuria in the 1st hour after injections with plasma sugar falling from 0.322 to 0.156 per cent, and none in the next hour with plasma sugar between 0.156 and 0.147 per cent. On February 19, 1918, glycosuria disappeared during the 2nd hour after injections with the plasma sugar somewhere between 0.294 and 0.170 per cent, for there was a bare trace of copper reaction in the urine of this hour and none immediately thereafter. Another comparison also can be made between these three dates, by consideration of the fact that the blood analyses during the injection periods were always performed 15 minutes after an injection, just before the next injection. They are thus minimum values, and though not establishing a threshold afford one basis of comparison. The table mentioned shows a change in sugar excretion on this basis. For example, at the 6th and 7th hours on August 6, with plasma sugars of 0.270 and 0.202 per cent, the glycosuria was 1.81 and 1.45 per cent. At the 6th and 7th hours on November 19, with plasma sugars of 0.356 and 0.370 per cent, the glycosuria was 0.28 and 0.55 per cent. At the 6th and 7th hours on February 19, with plasma sugars of 0.475 and 0.455 per cent, the glycosuria was 2.78 and 4.35 per cent. The differences exist likewise if the urine volume and quantitative sugar output are considered. In general, as the diabetes advanced and the dog was subjected to more prolonged hyperglycemia, the sugar threshold rose, but yet with the more severe diabetes there was a more active sugar excretion on February 19 than on November 19.

Dog B2-79, in the stage of severe diabetes over a year after operation, received 1 gm. of glucose per kilo per hour and did not show glycosuria when the plasma sugar had reached 0.230 per cent. In another experiment with 1.5 gm. per kilo per hour, the threshold of disappearance of glycosuria is shown in the table in the preceding paper.[11] There was very faint glycosuria from 3.45 to 4 p.m., while the plasma sugar was falling from 0.264 to 0.208 per cent, and none from 4 to 4.15 with plasma sugar of from 0.208 to 0.226 per cent.

[11] Allen and Wishart,[1] p. 450.

Carbohydrate Feeding.

The normal dog, No. C3-32, weighing 36 kilos, was given 1 liter of 50 per cent glucose solution by stomach tube. The results are shown in Table I of the ensuing paper.[12] The first trace of glycosuria found in frequent catheterizations came after 30 minutes, and the blood sample taken at this time showed 0.222 per cent of plasma sugar. Thereafter the blood sugar concentration fluctuated (as sometimes happens with large doses and particularly with nausea, due probably to irregularities of absorption), but heavy glycosuria occurred with much lower hyperglycemia, although the blood became concentrated as judged by hemoglobin and corpuscle percentage estimations.

Dog B2-00, partially depancreatized non-diabetic, weighing 14 kilos, received 3 gm. of glucose per kilo in 30 per cent solution by stomach tube. The first trace of glycosuria was obtained with a plasma sugar concentration of 0.192 per cent.

Dog B2-60, partially depancreatized non-diabetic, weighing 45 kilos, received 12 gm. of glucose per kilo of body weight in 40 per cent solution by stomach tube, and the threshold was found to lie between 0.202 and 0.222 per cent.

Dog B2-00, above mentioned, was fed 100 gm. of beef lung, 200 gm. of bread, and 75 gm. of glucose. The threshold of disappearance of glycosuria was determined between 0.154 and 0.149 per cent. The next day the same food was given with increase of glucose to 150 gm. The glycosuria was heavier, and a bare trace was still present after 6 hours with the plasma sugar at 0.128 per cent. 2 days later the same diet was given, and after 6 hours a faint trace of glycosuria persisted with plasma sugar of 0.130 per cent.

Dog B2-60, after removal of more pancreatic tissue but still not quite diabetic, received a feeding test with 100 gm. of lung, 800 gm. of bread, and 200 gm. of glucose, repeated on 4 different days. The threshold of appearance of glycosuria was determined in close agreement in all four tests as lying between 0.200 and 0.222 per cent. This is practically identical with the former test with pure glucose.

Dog B2-00 was fed the above diet of 100 gm. of lung, 200 gm. of bread, and 150 gm. of glucose when in the stage of mild diabetes. The threshold of appearance of glycosuria was determined between 0.145 and 0.152 per cent. 6 weeks later the same diet was fed, after hyperglycemia during most of the interval and some aggravation of the diabetes, and the first trace of glycosuria was found with plasma sugar of 0.204 per cent.

Dog B2-01, in the stage of mild diabetes, received a feeding test of 100 gm. of lung, 200 gm. of bread, and 30 gm. of glucose, repeated four times at 2 day intervals. The threshold of appearance of glycosuria was almost exactly 0.130 per cent on all four occasions, and was thus in keeping with the uniformly low thresholds in the early record of this dog. 3 months

[12] Allen, F. M., and Wishart, M. B., *J. Biol. Chem.*, 1920, xliii, in press.

later, the diabetes meanwhile having been well controlled by diet, the same test was repeated. The threshold of appearance of glycosuria was 0.135 per cent. The threshold of disappearance was not exactly determined, but a trace of glycosuria remained when the plasma sugar had fallen to 0.128 per cent. The same test was repeated 1 year later, when the diabetes was still under control and glycosuria or hyperglycemia had been permitted only in occasional short experiments during the interval. The threshold of appearance of glycosuria was between 0.141 and 0.147 per cent. The threshold was thus evidently rising. But the naturally low thresholds of this dog find their strongest contrast in the terminal stage with maximal severity of diabetes, when the plasma sugar was 0.322 per cent during fasting without glycosuria.[13]

Dog B2-02 was tested in the stage of mild diabetes after a meal of bread and soup, and glycosuria appeared with a plasma sugar concentration of 0.162 per cent.

Dog B2-88 was tested at a stage when the diabetes was mild but yet more advanced than that of the preceding dogs, so that chronic hyperglycemia was present on mixed diet. The fasting plasma sugar was 0.189 per cent. After a feeding test of 100 gm. of lung and 200 gm. of bread, it rose in successive analyses to 0.257 per cent without glycosuria, and 5 hours after feeding the first faint trace of glycosuria appeared with 0.286 per cent plasma sugar.

Protein Feeding.

As glycosuria from protein was taken as the criterion of severe diabetes, it follows that a renal sugar threshold on protein feeding can be determined only in severe cases. There is a considerable intermediate class of dogs (like a corresponding class of human patients) which have chronic hyperglycemia on protein diets, with no glycosuria even though the plasma sugar is constantly above the threshold of normal individuals. After a protein meal there is often a rise of blood sugar like that following carbohydrate in milder cases. As examples may be mentioned plasma sugar concentrations of 0.204, 0.222, 0.232, and 0.294 per cent in four dogs of this series under these conditions without glycosuria. These values do not establish thresholds, except that it can be said that the thresholds lie above these levels.

Examples of sugar thresholds on protein diets are given incidentally elsewhere, particularly under "Fat feeding." The question whether the protein itself has any influence upon the threshold in severe diabetes cannot receive a clear answer under the condi-

[13] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 600.

tions, for since these dogs cannot tolerate adequate protein diets it follows that in any prolonged experiments they must have been subjected to fat feeding, hyperglycemia, or marked undernutrition, any of which may possibly affect the threshold. In short experiments there is evidence that the threshold is approximately the same whether the hyperglycemia is produced by protein or by carbohydrate.

Special Influences Affecting the Threshold.

A. Fasting.—

Dog B2-00, partially depancreatized non-diabetic, as above mentioned under "Subcutaneous glucose injections," received 3 gm. of glucose per kilo and showed slight glycosuria with 0.163 per cent plasma sugar. After the dog had fasted for 1 week the same subcutaneous dose was repeated, and the plasma sugar reached 0.238 per cent without glycosuria.

Dog C3-22, with severe diabetes which had been partially controlled for 6 weeks with diets very high in fat, showed hyperglycemia as high as 0.256 per cent without glycosuria. After 5 days of fasting, a feeding test fixed the threshold of appearance of glycosuria at 0.159 per cent.

The different effects of fasting possibly have some relation to the preceding diet.

B. Excess of Sugar.—Under "Fasting plasma sugars" mention was made of the fact that the high thresholds characteristic of severe diabetes are generally found after long standing hyperglycemia, and the latter is with some reason regarded as a factor in raising the threshold, though some examples were given of high thresholds after briefer hyperglycemia. A normal cat subjected to subcutaneous glucose injections through many months[14] developed a remarkable pathologic condition, particularly with regard to nervous manifestations, and at the close a blood sugar concentration of 0.539 per cent was found without glycosuria. The simple osmotic disturbance of the subcutaneous injections may have been responsible for the high sugar threshold as well as for the other pathological symptoms. Dogs B2-00 and B2-01, frequently mentioned in this and preceding papers,[15] were among numerous animals subjected to the highest possible carbohydrate diets through months and years, without elevation of the thresh-

[14] Allen,[3] pp. 159, 164, 168. Microphotograph of adrenal medulla, Fig. 1.
[15] Allen, F. M., *J. Exp. Med.*, 1920, xxxi, 396; Allen,[9] pp. 562, 564.

old before they became diabetic. Allowance must be made for the fact that the hyperglycemia in such animals is generally brief, and is never actually continuous over any long time unless the animal becomes diabetic. A considerable degree and duration of hyperglycemia are occasionally attained in certain dogs barely on the edge of diabetes, as Dog B2-86, previously described.[16] After removal of about $\frac{4}{5}$ of the pancreas on April 7, glycosuria was maintained almost daily by enormous glucose diets. In the feeding test with 400 gm. of bread and 200 gm. of glucose on May 4, the urine taken at frequent intervals first showed sugar about the end of the first $\frac{1}{2}$ hour, with plasma sugar of 0.196 per cent. From the table given for May 13 it may be judged that the threshold on that date was not far different. The threshold therefore seems to be slightly elevated as compared with the normal; but as the dog was old and his threshold before the pancreas operation had not been determined a conclusion is unsafe. Furthermore, the tables referred to furnish evidence that important hyperglycemia must have been present for about a month, yet the threshold was not so high as that of severely diabetic dogs a few days after operation.

C. Fat Feeding.—Most of the severely diabetic patients and animals with high sugar thresholds have been on diets containing considerable fat for longer or shorter periods. This factor cannot be excluded in any of the high thresholds of severely diabetic animals mentioned thus far, except in a few cases of fasting. The thresholds have been noticeably high in the animals receiving the highest fat diets, and one of the highest in the series was observed under these conditions.[17] Here the threshold was evidently above 0.4 per cent, and the suggestion was made that excessive fat feeding may affect the kidney function injuriously.

Some indications of a direct influence of fat seemed to be furnished by Dog B2-79. The high thresholds previously mentioned (p. 135) were obtained after very high fat diets (up to 300 gm. of lard or suet daily for over a month). Then for 5 days the only diet was 500 gm. of lung daily without glycosuria. At the end of this time a meal of 1 kilo of lung was given, and glycosuria began when the plasma sugar reached 0.159 per cent. The high

[16] Allen,[15] p. 385.

[17] Allen, F. M., *Am. J. Med. Sc.*, 1917, cliii, 348, 352.

fat diets were then resumed for 3 days, after which a fasting plasma sugar of 0.200 per cent was found without glycosuria. After 2 months more of diets consisting predominantly of fat, a protein-feeding test showed a threshold of 0.334 per cent.

Several observations indicate that a single feeding with fat has no demonstrable influence on the sugar threshold. No prolonged experiments with fat feeding were performed in normal or mildly diabetic animals.

D. Acidosis.—This was one of the factors considered by Fitz[18] in his study of the renal function in diabetes. It can be excluded in regard to most of the high thresholds and also the fat diets of this series, because the dogs were free from acidosis. Dog B2-79 had slight acidosis with some of the thresholds mentioned, and Dog C3-56 had more marked acidosis at certain times. Sugar thresholds were not obtainable in coma cases in dogs because of the continuous glycosuria.

Considerable hyperglycemia without glycosuria may be found in experiments with large intravenous injections of either acetone bodies or mineral acids, such as will be described elsewhere, but the renal changes can scarcely be classified as typical of acidosis because of the chance that they are due merely to this mode of administration of a toxic foreign substance.

E. Cold.—The opportunity was taken to determine approximately the sugar thresholds of several dogs used for experiments with cold to be described elsewhere,[19] but this degree of cold seemed to produce no important alterations as compared with the thresholds of the same dogs at ordinary temperatures.

F. Exercise.—No sugar thresholds were determined in exercise experiments, but the normal dog, No. B2-90, with exercise alone attained a plasma sugar concentration of 0.256 per cent without glycosuria, which is probably higher than the threshold of any normal dog under ordinary conditions.

G. Infections.—A normal dog shortly before death from rabies had a plasma sugar of 0.450 per cent without glycosuria. Sugar thresholds were not determined in dogs with ordinary infections and would probably have little singificance because of the varying grades of renal injury to be expected in such cases.

[18] Fitz, R., *Arch. Int. Med.*, 1917, xx, 809.

[19] Allen, F. M., Experimental Studies on Diabetes, Series II, Paper 7.

H. Hemorrhage.—A normal dog weighing 10.8 kilos was bled rapidly from the femoral artery to the amount of 500 cc. The preparation had been made in advance so that there was no pain or struggle. The plasma sugar at the beginning of bleeding was 0.121, at the end 0.238 per cent, without glycosuria. The hyperglycemia of hemorrhage is well known, and the apparently elevated threshold agrees with observations of Wilenko[20] and of Epstein and Baehr.[4]

I. Unilateral Nephrectomy.—

Dog D4-69 in January, 1917, underwent removal of the left kidney, followed by partial pancreatectomy not quite sufficient to produce diabetes. In March, on diets of bread and soup with 200 gm. of glucose, plasma sugar concentrations as high as 0.264 per cent were observed without glycosuria. No reason was known for the apparently elevated threshold except the kidney removal.

J. Epinephrine.—Pollak[21] discovered that repeated injections of epinephrine diminish the renal permeability for sugar.

The normal dog, No. C3-92, weighing 17.6 kilos, on May 16, 1916, received 10 cc. of Parke-Davis adrenalin ($\frac{1}{1,000}$) solution subcutaneously. The threshold of appearance of glycosuria was determined at 0.152 per cent plasma sugar, the threshold of disappearance at 0.118 per cent. On May 23 the same dose was repeated, and the threshold of appearance of glycosuria was approximately 0.380 per cent. The dose was repeated May 29, and the plasma sugar rose to 0.418 per cent without glycosuria. Lard was fed on May 23 and white clay as a control on May 29. Probably neither of these influenced the threshold, for several similar examples could be cited which are free from such factors; but it seems true nevertheless that the diuretic activity and other accessory conditions influence the sugar threshold with epinephrine. Especially in fasting animals plasma sugars as high as 0.278 per cent have been seen with the first dose of epinephrine without glycosuria.

K. Drugs.—Narcotics and other drugs which produce hyperglycemia and glycosuria (supposedly due largely to asphyxia) at the same time impair the renal function for sugar. For example, a normal dog was deeply narcotized with magnesium chloride intramuscularly, and when near death was partially revived with calcium chloride intravenously. A trace of sugar

[20] Wilenko, G. G., *Arch. exp. Path. u. Pharmakol.*, 1912, lxviii, 297.
[21] Pollak, L., *Arch. exp. Path. u. Pharmakol.*, 1909, lxi, 376.

appeared in the urine when the plasma sugar reached 0.500 per cent. Figures almost as high have been found with the huge doses of morphine necessary for deep narcosis of dogs. Examples of such drug action might be multiplied, probably without much significance.

L. Pancreatic Function, or Severity of Diabetes.—One of the questions of chief interest was a possible relation between the renal function and the internal pancreatic function. It may be noticed under "Fasting plasma sugars" that the dogs which developed a high renal threshold very early after operation were those which had very small pancreas remnants and correspondingly severe diabetic tendencies. On the other hand, clinical literature contains occasional statements that some cases of diabetes begin with glycosuria with little or no hyperglycemia. To obtain information whether complete lack of the pancreatic function may make the kidneys more or less permeable for sugar, observations of the threshold of appearance of glycosuria were carried out on two totally depancreatized dogs.

Dog C3-01, a collie aged 3 years, thin at a weight of 15 kilos, after 24 hours of fasting before operation had plasma sugar of 0.099 per cent. In the midst of the operation, when the entire pancreas had been dissected free except that its duct and principal blood vessels were not yet severed, the plasma sugar was 0.236 per cent without glycosuria. On removal from the table 15 minutes later glycosuria was still absent and remained so for $2\frac{3}{4}$ hours; then with the appearance of slight glycosuria the plasma sugar was 0.270 per cent. At the end of 5 hours the plasma sugar was still 0.270 per cent, with 1.6 per cent urinary sugar. On the following days, with the usual heavy glycosuria during fasting, the plasma sugars ranged between 0.3 and 0.5 per cent.

Dog C3-60, a mongrel, in medium condition at a weight of 12.5 kilos, had plasma sugar of 0.208 per cent immediately after total pancreatectomy, without glycosuria. The exact threshold was not determined, but 4 hours later there was heavy glycosuria with plasma sugar of 0.300 per cent.

Doubtless the state of nutrition, the anesthesia, and other accidental factors influence the exact threshold and the interval after operation at which glycosuria begins, but these and similar observations seem to exclude any abnormally low renal thresholds at any stage following total pancreatectomy.

DISCUSSION.

At least three groups of causes may be imagined for the alterations of renal function in diabetes; namely, (*a*) direct renal and vascular injuries, (*b*) general metabolic conditions, and (*c*) influences associated specifically with the diabetes.

(*a*) Clinical diabetes is not infrequently associated with some degree of nephritis or arteriosclerosis, and even where these are not evident there is a possibility that the same infection or intoxication which damaged the pancreas may have left some anatomic or functional injury in the kidney. Animal experiments offer the opportunity either of studying diabetes without these possible complications, or of producing different forms of renal injury for investigation. Though the supposition of an adrenal element in diabetes is opposed by weighty facts and supported by none, the use of epinephrine as a drug gives an interesting illustration of a toxic renal or vascular alteration. When the acute effects have subsided, it is questionable whether the finest study can reveal any anatomic changes, and the animals are certainly free from albuminuria and the ordinary clinical symptoms of nephritis. Yet there is an elevation of the sugar threshold which is far greater than can be accounted for by the brief hyperglycemia, and which is either permanent or at least of considerable duration. Animals which have undergone removal of $\frac{1}{2}$ to $\frac{3}{4}$ of their total renal tissue offer some points of interest not only for the sugar threshold in the old sense but also for their sugar excretion by Benedict's new method, for determining to what extent the latter represents general carbohydrate metabolism or merely a function of the kidney itself.

(*b*) Some general metabolic alterations might consist in accidentally associated endocrine disorders, which, notably in the case of the hypophysis, are recognized as affecting renal function. The nutritive state is another possible factor. The so called famine or war edema is presumably similar to that of many diabetic cases. The latter is usually accompanied by a high renal threshold for both sugar and salt, but the relation is not known to be invariable; equally high thresholds are found in cases without edema, and the etiology may be independent.

Alterations in blood volume are a factor adequately discussed by authors already quoted. A possibility not much considered heretofore is an influence of food upon renal function. Barrenscheen[22] claimed that an "oatmeal cure" may diminish the permeability of the kidneys for lactose, though the fact itself and the interpretation, whether due to carbohydrate, salt, or something else, may bear further investigation. On the other hand, there is abundant evidence that in the declining stage of a glycosuria there is ordinarily a lowering of the renal threshold, in the sense that sugar continues to pass into the urine at a level of plasma sugar concentration distinctly lower than that necessary to produce glycosuria in the first place. One hypothetical explanation may be suggested by the well known Armanni or Ehrlich phenomenon of glycogen deposition in certain segments of the renal tubules. If these deposits be accepted as representing sugar absorption by the tubule cells, it is conceivable that cells thus saturated may either continue for a time to discharge sugar, or (more probably) that they may fail to resorb sugar as actively as usual from the glomerular filtrate, and thus the prolongation of glycosuria might be explained under either of the theories of renal secretion. A possible influence of fat feeding is also suggested by other evidence. Chemical analyses in the literature indicate that the kidney is one of the organs in which fat is deposited during lipemia. Especially with heavy lipemia this can be confirmed microscopically, and it is interesting that the cells which stuff themselves with fat are the same ones which are filled with glycogen in glycosuria. The two may be combined; carmine stains then show the glycogen granules to be distributed around, not in, the vacuoles, and the latter are shown by Sudan stains to be fat. This fat infiltration accords with the traces of fat found in the urine with heavy lipemia, and can be interpreted according to either theory of renal secretion. If fat feeding causes a diminution of renal permeability for sugar, it may be associated in some unknown way with these morphologic changes or due to invisible alterations in cell membranes.

(*c*) One condition in diabetes which may affect the kidneys is the acidosis. This supposition is supported by the well known albuminuria and showers of casts appearing as precursors of coma.

[22] Barrenscheen, H. K., *Biochem. Z.*, 1912, xxxix, 232.

This can, however, be easily excluded as a sole cause by the fact that dogs and most human patients studied in sufficiently early stages show a considerable elevation of the sugar threshold without acidosis. The typical onset of diabetes in all but the most acute cases seems to be by a gradual rise of blood sugar, frequently traceable through months in both animals and patients, before glycosuria appears; there is generally continuous hyperglycemia in the next stage of occasional or "alimentary" glycosuria, and generally next a period of continuous glycosuria without acidosis. In most cases at any of these stages the sugar threshold is found to be already high.

This elevation of the threshold may be attributed to the prolonged hyperglycemia itself, a common notion being that the kidney by habituation comes to "tolerate" glucose. This explanation may contain much truth, but yet it appears that a high threshold may develop very early in severe diabetes and may be absent after more intense and prolonged hyperglycemia and glycosuria in normal or mildly diabetic animals. Also, if this elevation is a mere habituation, it might be expected to be lost after removal of the cause, but in the writers' experience severely diabetic patients and animals have shown characteristically high sugar thresholds after a year or more of continuously normal blood sugar. The reason for the low thresholds of exceptional clinical cases is also not clear.

A specific relation of the renal and pancreatic functions is not demonstrable in the sense of any increased readiness of sugar excretion following total pancreatectomy. An opposite view is sometimes entertained, that the elevated sugar threshold is to be regarded as a protective mechanism for saving sugar to the body. It might further be considered plausible that this device is more needed and called more into play with increasing severity of the diabetes. There is no real disproof of this assumption. It is true furthermore that some extra utilization of carbohydrate is probably forced by any obstruction of excretion, according to the principle of the metabolism of plethora, as long as any appreciable power of utilization is retained by the organism. It was thus found in the preceding paper that renal impermeability confuses judgment of the urinary findings in glucose tolerance tests, particularly intravenous tests of short duration. It has less influence

upon feeding tests or any tests of long duration, because the blood sugar rises high enough to cause heavy glycosuria even at the higher threshold. Theoretically the interpretation of the elevated threshold as a teleologic adaptation is an unproved and improbable assumption. The chief error which can be refuted is the supposition that the renal impermeability is of any real benefit to the diabetic organism in a practical sense. This involves the usual confusion between diabetes and glycosuria, and is closely allied to the discredited clinical practice of giving arsenic, uranium, and other drugs which perhaps to some extent suppress glycosuria by injuring the kidneys. Two important principles, which are perfectly clear in animal experiments, are equally applicable clinically. First, diabetes is deficiency of the internal secretion of the pancreas, and this deficiency cannot be compensated by any mere blocking of renal excretion. An extreme illustration is in totally depancreatized dogs, which die all the more quickly when their sugar is dammed back by nephrectomy or ureteral ligation. Second, the essential trouble to be combated in diabetes is not the mere loss of food material in the urine. The saving by renal impermeability, and also the metabolism of plethora induced by increased sugar concentration in the blood, can readily be equalled in the milder forms of diabetes by increasing the diet, and as the latter process is harmful the former may be expected to prove so likewise. In severe diabetes the damming back of metabolic products which cannot be utilized is a still more serious complication, as above mentioned. Some clinical illustrations of the above statements are found in the symptoms which occurred with trivial glycosuria and plasma sugar of 0.73 per cent in a patient with combined diabetes and nephritis,[23] and the occurrence of typical diabetic symptoms and complications in patients with hyperglycemia with little or no glycosuria because of a high renal threshold.[24]

The chief criticism of the results contained in this communication is that they possess only the suggestiveness which is more proper to clinical observations, and could not be followed up with

[23] Allen, F. M., Stillman, E., and Fitz, R., Total dietary regulation in the treatment of diabetes, Monograph of The Rockefeller Institute for Medical Research, No. 11, New York, 1919, Case 61, 413.

[24] Mitchell, J. W., *Am. J. Med. Sc.*, in press.

conclusive methods and controls to establish causes and laws in the manner to be expected from animal experiments. The following deductions are possible concerning some of the points chiefly in view.

CONCLUSIONS.

1. A diminished renal permeability for glucose, in the sense of a raised threshold according to tests with Benedict's copper solution, is definitely proved as the rule in diabetic animals. As the same rule holds for the great majority of human diabetics, this demonstration furnishes one more point of similarity between the experimental and clinical conditions.

2. Various possible causes for the elevation of the threshold are discussed. The prolonged excess of sugar in the blood may be an important factor, but some considerations seem to oppose it. Certain observations suggest that high fat diets may raise the sugar threshold in diabetes even without acidosis. The renal function may be affected by various extraneous causes, of which the elevation of the threshold by epinephrine is one interesting example.

3. No interrelation of the renal and pancreatic functions is demonstrable in the sense of an increased readiness of sugar excretion even in totally depancreatized animals. An elevation of the threshold seems to be connected particularly with severity of the diabetes, but a teleological interpretation of this as a protective mechanism for saving sugar to the body is considered improbable.

DETERMINATION OF IODINE IN CONNECTION WITH STUDIES IN THYROID ACTIVITY.

THIRD PAPER.

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In 1912 I published a method for the determination of iodine in the presence of the other halogens and organic matter (1). In 1914 the method was modified in part (2), and during the past 6 years has been in constant use. Recently it has become necessary to determine the iodine content of animal tissues, and, as no method yet published was suitable for this work, attempts were made to refine the method published in 1914, in order to make it applicable for the determination of the iodine in blood and tissue.

The faults of the iodine method, previously described, apply entirely to the solution obtained after fusion with sodium hydroxide. The fusion originally described is still used without change.

In trying to improve the accuracy of the method, it was first necessary to secure a perfect blank in the absence of iodide. It was pointed out in a previous paper (2) that the presence of salts assists rather than interferes with the liberation of iodine from potassium iodide by an oxidizing agent. Blanks were therefore prepared, using sodium phosphate made by neutralizing the amount of sodium hydroxide required by the method with phosphoric acid. The neutralization was carried to the turning point of methyl orange. When such a solution was oxidized with bromine, then cooled, and phosphoric acid, potassium iodide, and starch were added, a perfect blank sometimes was obtained, but the blue color rapidly developed if the solution was allowed to stand.

In the determination it is essential to control the hydrogen ion concentration during the boiling with bromine. This is done by neutralizing the alkaline solution with phosphoric acid until methyl orange is just pink. This acidity, however, is not great enough for the final reaction between potassium iodide and the iodic acid resulting from the oxidation with bromine. It is necessary to add about 5 cc. of 20 per cent phosphoric acid at the time the potassium iodide is added. As we failed consistently to secure a perfect blank after oxidation with bromine, it appeared probable that the phosphoric acid which was used to acidify the solution at the time the potassium iodide was added was the cause for the liberation of iodine, and attempts were therefore made to obtain a perfect blank with nothing present except water and phosphoric acid. The test as finally adopted is as follows: 1 gm. of potassium iodide is placed in a dry flask. 15 cc. of phosphoric acid, 85 per cent, syrupy, are added to the potassium iodide. This produces a yellow color which, however, is not free iodine. The flask is allowed to stand for 15 minutes. 100 cc. of water are added. This will cause a partial or complete disappearance of the yellow color. After starch is added the solution should remain colorless. With the phosphoric acid available on the market it is rarely possible to obtain a perfect blank after such a procedure.

Since within the phosphoric acid itself there is a source of error causing high results, it is necessary to treat the acid so that this oxidizing action may be removed. This may be accomplished by diluting the phosphoric acid with four equal volumes of water, placing in a large beaker, and boiling the solution after the addition of a suitable amount of aluminum in the form of strips. It was found that the reduction proceeds slowly and requires prolonged boiling. It is possible to reduce all oxidizing agents so that when 25 cc. of the reduced acid are added to 1 gm. of potassium iodide, the solution is allowed to stand 15 minutes, and water and starch are added, a perfect blank will be obtained which will remain colorless for at least 15 minutes. When phosphoric acid which has been so reduced is used, the method is found to give very much more consistent results, and the solutions will remain colorless after titration for as long as from 30 to 45 minutes. It is unnecessary to reduce the phosphoric acid used to

neutralize the sodium hydroxide. Treatment of the solution with bromine destroys the oxidizing action of the impurities in the phosphoric acid.

In the original method talcum was recommended for the rapid removal of bromine from the solution. It was found, however, that when the talcum settled to the bottom of the titration flask the solution turned blue, suggesting that the talcum, although it caused a rapid expulsion of bromine from the solution, had retained a small amount which was absorbed in some manner and was not reduced by boiling with salicylic acid. Another disadvantage was the cloudiness of the solution. A solution turbid with talcum is very much more difficult to titrate than a water-clear solution. Some substitute for talcum was urgently needed. Many different substances were tried among which may be mentioned sand, brick, granite, pumice stone, capillary glass tubing, paper fibers, asbestos fibers, powdered charcoal, powdered glass, powdered porcelain, powdered hard coal, coke, and eventually hard coal in lumps of about 0.5 cm. in diameter. It was found that some of these agents assisted slightly in producing rapid boiling, but they all, except the paper fibers, retained traces of bromine which liberated iodine, giving high results. The paper fibers caused a slight reduction of the iodic acid.

Hard coal in lumps possesses a unique power to induce rapid boiling, but it also absorbs small amounts of bromine, so that after the titration is finished the solution in the immediate vicinity of the lump of coal turns blue, even in a flask which should be a perfect blank. In order to prevent high results from the oxidation by the bromine held in the coal, it was decided to remove the piece of coal after the solution had been boiled, and before titration. In order to accomplish this, two pieces of coal were tied together with a piece of thread. This thread could be picked up easily with a glass rod terminating in a hook. When pieces of coal tied together with a thread are used a number of times, added iodine cannot be quantitatively recovered, and it was found that the explanation for the low results is the reduction of iodic acid by the thread. It was a simple matter to change the thread to a glass hook which was passed through a hole in the lump of coal made by a dental drill. For some time coal which was removed by means of a glass hook was used, the coal

being washed between determinations and used a large number of times. This, however, was found impracticable, since there is a slow oxidation of the coal by the bromine, resulting in a softening and disintegration which eventually has the effect of reducing iodic acid and causing low results.

Since coal can be prepared in any desired amount with trivial trouble and expense, it was decided to use a fresh piece of coal for each determination. The method now employed is to use a piece of coal about 0.5 cm. in diameter and, after the solution has been boiled and cooled, it is removed by means of a small shovel made from a piece of sheet silver attached to a glass rod. This procedure is very easily carried out, the coal does not reduce iodic acid when used but once, and if it is removed from the solution a perfect blank can be obtained in the absence of iodine.

The method now possessed a high degree of accuracy except for the possible presence of substances other than iodic acid which were in the water or resulted from the solution of the fusion. In attempting to remove such oxidizing agents the solution was boiled with a considerable excess of sodium bisulfite and it was found that when iodine is present in amounts of from 5 to 10 mg., which is the upper limit of amounts that are desired to be determined by this method, the boiling in acid solution with sodium bisulfite does not result in the loss of any iodine. Boiling with sulfite reduces all nitrites and other oxidizing agents and prepares the solution for oxidation. Oxidation of such a solution with bromine causes a quantitative production of iodic acid.

Another point which is essential to control is the hydrogen ion concentration of the solution. This we find to be of greatest importance. In the presence of too high a concentration of hydrogen ion, hydrobromic acid will react with iodic acid, causing low results. Even 5 cc. of 50 per cent phosphoric acid in excess may cause an appreciable loss of oxidizing power due to reduction of iodic acid. It was eventually found that titration to the first pink color with methyl orange, after which 5 cc. of 20 per cent phosphoric acid are added, produces a proper acidity for both the reduction with sodium bisulfite and the subsequent boiling with bromine.

In the original method it was recommended to use a 10 per cent solution of potassium iodide. All commercial potassium

iodide will liberate traces of iodine on long standing in aqueous solution. This iodine could be removed with thiosulfate, but the addition of potassium iodide in solid crystalline form to the solution has been found to be the most convenient method for the addition of this salt.

The last point which requires control is the length of time of boiling the solution. It has been found that the solution may be boiled in the presence of sodium bisulfite until a large part of the water has been evaporated without loss of iodine. The solution must be boiled 10 minutes as a minimum length of time, in order to remove all traces of sulfurous acid. The oxidation with bromine is best carried out in a solution of a volume of 200 cc. It is best therefore to have sufficient water present before boiling off the sulfurous acid so that at least 10 minutes are necessary to bring the volume to 200 cc. This volume is marked on each flask. If more time is required to reduce the volume of the solution to the mark it is without effect on the subsequent determination of the iodine. The only essential point is that each flask boils at least 10 minutes.

Although there is a wide limit to the time for boiling with sulfite, it is essential to regulate closely the time of boiling with bromine. If the boiling with bromine is prolonged after the excess has been removed from the solution, low results are produced. A series of experiments has shown that the best time for boiling with bromine is 5 minutes after the solution is colorless. It requires approximately 3 minutes for the bromine to be expelled from the solution after boiling begins, and if the boiling is continued for 5 minutes more a perfect blank will result. As a further precaution, however, salicylic acid is added just before the flask is cooled. Although this may be unessential in most cases, its adoption is a safeguard which assures a uniform removal of the last traces of bromine from all determinations.

With the adoption of these six modifications of the original method, the determination of iodine after fusion in sodium hydroxide can be made with a high degree of accuracy. The method is of the greatest value, however, in the determination of minute amounts of iodine. The water-clear solution resulting by elimination of talcum permits a titration of amounts measured in hundredths of a cubic centimeter, and the removal of all

substances which retain bromine and therefore cause slight liberation of iodine permits the determination of small traces of iodine with a much higher degree of accuracy than before. For example, it has been found that talcum, even after boiling for 10 minutes, will retain bromine equivalent to as much as 0.08 cc. of 0.005 N thiosulfate. This amount of iodine is approximately that found as the normal iodine content of 100 cc. of blood, so that any results which have been obtained in the past with talcum are questionable, at least to within 0.05 to 0.08 cc. of 0.005 N thiosulfate. The method in detail as now employed in our laboratories is as follows:

The first step is the destruction of organic matter and the retention of the iodine as sodium iodide. This is accomplished by fusion with sodium hydroxide in a nickel crucible. In order to maintain a proper degree of temperature and to prevent loss of iodine by volatilization, the crucible is heated indirectly by placing inside a larger crucible, the bottom of which is covered with a layer of sand 0.5 cm. in thickness. The details of the construction of the heating apparatus have been described elsewhere (2). The supporting cylinder is 9.4 cm. in diameter and 30 cm. high; the cross bars which support the large crucible are 7.5 cm. from the top. The larger crucible is of iron and is 7.8 cm. in diameter. The smaller one is of pure nickel and is 5.9 cm. in diameter. The burner is preferably a 15.6 cm. (No. 3) Meker burner.

For the determination of iodine in thyroid preparations it is best to use not more than 0.5 gm. of material. Whether this is in the form of a dry powder, a solution, or a moist precipitate on a filter paper, the same procedure is carried out. The material is placed in a 5.9 cm. nickel crucible and moistened with a few drops of 30 per cent sodium hydroxide; 5 to 10 gm. of stick sodium hydroxide which has been broken into small pieces are added and the crucible is placed on a hot plate until the excess of water is evaporated and the contents have a thick, syrupy consistency. If but little organic matter is present, there is a tendency for spattering of fine drops during the evaporation of the excess water. If some organic substance is dissolved in the solution this spattering is prevented. A small amount of lactose is suitable and sufficient for this purpose.

For the fusion of the organic matter with the sodium hydroxide it is necessary to heat the bottom of the large crucible to a red heat. If the crucible is heated too much the fusion in the small crucible will creep up the sides and sodium hydroxide will volatilize with loss of iodine. If the large crucible is heated insufficiently the destruction of organic matter will not be complete. However, there is a wide range of temperature between the two limits and after a little experience no difficulty is encountered.

When the sodium hydroxide is first heated in the presence of water considerable foaming is produced. This, however, does not extend more than half way up the sides of the crucible. As the heating continues the foaming becomes less and after from 5 to 10 minutes the melt settles to the bottom. Bubbles continue to be given off for some time (5 minutes or so), depending on the nature of the organic matter.

When the melt has settled to the bottom and only a few bubbles of gas are being liberated, the small crucible is removed with crucible tongs and partially cooled by agitating the contents with a rotary motion. This will also remelt and carry to the bottom any particles of the fusion which have solidified on the cooler sides of the crucible.

5 to 10 mg. of potassium nitrate are now added. This oxidizes the remaining organic matter and causes a liberation of bubbles. If only a few bubbles appear, a second addition of from 5 to 10 mg. of potassium nitrate will not cause a further liberation of bubbles and the oxidation of the organic matter is complete. If the second addition of nitrate causes a further oxidation, repeated additions of from 5 to 10 mg. of the nitrate are made until no more bubbles of gas are produced by the addition of the nitrate. The melt is now poured into the shallow cover of the 5.9 crucible and allowed to cool.

The entire time required for the fusion is from 10 to 15 minutes. It is most convenient to use two crucible-supporting cylinders and to carry on two fusions at the same time.

When the melt and crucible are cool, they are placed in a beaker of from 600 to 800 cc. capacity and 125 to 150 cc. of water are added. The beaker is then placed on a hot plate. After the melt is dissolved it is transferred to a 500 cc. Erlenmeyer flask.

It should be a colorless, clear solution with a volume of about 200 cc. To the solution 5 cc. of 20 per cent sodium bisulfite and just two drops of a saturated water solution of methyl orange are added.[1] The solution is cooled by immersing the flask in cold running water. When it is cool, 85 per cent phosphoric acid is added by allowing the acid to run directly into the flask from a pipette or syphon having a small delivery tube. The flask is vigorously and constantly shaken with a rotary motion to expel the carbon dioxide. As the indicator begins to turn pink the neutralization is finished slowly, and the addition of acid is stopped at the first definite change of the indicator to pink. 5 cc. of 20 per cent phosphoric acid and a small piece of hard coal, about 0.5 cm. in diameter, are added. The volume is adjusted to about 250 cc. and the water is boiled on a hot plate for at least 10 minutes, and longer if necessary, until the volume of the solution is about 200 cc. After the flask has been cooled in water bromine is added and the solution shaken until the bromine imparts a distinctly yellow color. This is essential, as the addition of too little bromine will prevent the subsequent determination of iodine. The flask is again placed on a hot plate and the solution is boiled, the time when all visible bromine is expelled being noted. This will require about 3 minutes of actual boiling. The solution is boiled for just 5 minutes after it is colorless. It is then removed from the hot plate, a few crystals of salicylic acid are added, and the flask is immersed in cold water. The volume of the solution after boiling should not be less than 175 cc. as the high concentration of the salts makes the end-point less sharply defined.[2] 5 cc. of reduced 20 per cent phosphoric acid and about 1 gm. of pure potassium iodide crystals are added.

[1] The sulfite and bromine destroy the color of methyl orange if only two drops are added. If more than two drops are added the solution may retain a pink color.

[2] Experience has shown that practically all samples of distilled water take up small amounts of iodine. As this reducing action is destroyed by boiling with bromine this source of error is entirely controlled under the conditions of the method described above. However, a considerable error may result in the determination of iodine if any distilled water is added after the bromine has been boiled out of the solution.

The liberated iodine is now titrated with 0.005 N sodium thiosulfate.[3] The titration is finished with the aid of the blue

[3] The best strength of sodium thiosulfate for amounts of iodine ranging from 0.1 to 5 mg. is 0.005 N. The stability of the thiosulfate solution depends in large part on its manner of preparation. The most satisfactory method is to boil distilled water for at least 30 minutes, leaving the final volume about 4 liters. 5.25 gm. of sodium thiosulfate crystals are added to about 1 liter of this boiled water and this solution is boiled for about 30 minutes. The solution is cooled and made to 4 liters with the boiled water and is protected from carbon dioxide by a sodium hydroxide tube. This solution must be restandardized frequently. A convenient method proposed by Hunter is to prepare a solution of potassium acid iodate which is equivalent to a known weight of iodine. The strength of any sample of thiosulfate is readily found by titrating the iodine liberated by the acid iodate solution, which retains its strength indefinitely. The iodine equivalent of the potassium iodate is found as follows:

Prepare a 0.1 N solution of potassium acid iodate, KIO_3HIO_3, by dissolving 3.249 gm. of the salt in 1 liter of water. This solution diluted twenty times will be approximately 0.005 N. Dissolve a known weight of pure iodine (approximately 1 gm.) in 1 liter of water containing from 5 to 6 gm. of sodium hydroxide. Dilute this ten times. 1 cc. of this solution will contain 0.1 mg. of iodine. Measure 25 cc. of this solution into a 500 cc. flask and dilute to 200 cc.; add 10 cc. of 30 per cent sodium hydroxide; and neutralize as in the determination with 85 per cent phosphoric acid to a definite pink color with methyl orange. Add 5 cc. of 20 per cent phosphoric acid, a small piece of hard coal, 5 cc. of 20 per cent sodium bisulfite, place the flask on a hot plate, and boil for at least 10 minutes. Cool, add bromine, and boil as described in the determination. Add salicylic acid, cool, then add 5 cc. of 20 per cent reduced phosphoric acid, 1 gm. of potassium iodide crystals, and titrate the liberated iodine with approximately 0.005 N thiosulfate. This will establish the relation between "original iodine" and the 0.005 N thiosulfate, and from this the iodine equivalent of the potassium acid iodate can be found as follows:

Add a known volume, 10 or 15 cc., of the acid iodate solution to a 150 cc. flask. Add 0.25 gm. of potassium iodide crystals, five drops of a 20 per cent reduced phosphoric acid solution, and titrate with the thiosulfate, using starch at the end. When a small amount of iodic acid is in a solution which contains but a small amount of salts, the reaction with potassium iodide is retarded and the end-point of the titration with thiosulfate is uncertain. In the original method the volume of water present with the potassium acid iodate was about 150 cc. and to this sodium chloride was added in order to increase the distinctness of the end-point. It has been found that the salt is a source of error and that more accurate results are obtained by not diluting the iodate solution. The potassium iodide added should be reduced to a minimum, as large amounts will cause a pink-colored end-point instead of blue with starch.

starch-iodine color. For this a 0.5 per cent solution of soluble starch is recommended (3).

The most convenient procedure if a number of determinations are made each day is to use six or seven crucibles, each marked with a letter for identification. Each crucible has a corresponding 500 cc. Erlenmeyer flask marked with the same letter. The crucibles are used in rotation. Frequent blanks should be made on all reagents used, and the reduced phosphoric acid should be tested at intervals with potassium iodide.

Accuracy of the Results.

The following determinations were made with iodine in the form of potassium iodide added to water. Titrations were made with 0.005 N thiosulfate.

Amount found.			
1.0 mg. iodine added.	0.5 mg. iodine added.	0.3 mg. iodine added.	0.1 mg. iodine added.
mg.	*mg.*	*mg.*	*mg.*
1.008	0.501	0.299	0.102
1.008	0.500	0.300	0.102
1.000	0.501	0.302	0.101
1.000	0.502	0.302	0.101
1.000	0.503	0.299	0.102
1.000	0.502	0.298	0.105
1.004	0.504	0.299	
1.004	0.504	0.299	
1.004			
1.007			
1.006			
1.002			
1.004			
1.004			
1.007			
1.007			
1.000			
1.000			
1.000			
1.000			
1.000			
0.994			
0.994			
0.993			
0.993			

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DETERMINATION OF IODINE IN BLOOD AND IN ANIMAL TISSUES.

FOURTH PAPER.

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Although the refinements outlined in the previous paper permit the determination of minute amounts of iodine, the method cannot be applied directly with blood and tissues because the iodine content of 0.5 gm. or even 1 gm. of the material is much too small to be detected. However, the only additional requirement to the method is the destruction of a large amount of organic matter without loss of iodine. The first attempt was to hydrolize the proteins of blood and tissue with 5 per cent sodium hydroxide in an autoclave at 200°. The solution was acidified and filtered. Attempts were then made to distill the iodine from the solution after the addition of an oxidizing agent. For this purpose chromic acid, copper, iron, and arsenic were tried, and it was found that although the iodine was liberated it was not quantitatively distilled.

The next attempt to destroy the organic matter was by means of a fusion with nitrate. This resulted in excessive heating, the organic matter frequently igniting. As this was always accompanied by loss of iodine, it was found impracticable to destroy the organic matter with oxidation.

Attempts were then made to remove the organic matter simply with a destructive distillation, and it was eventually shown that in the presence of about 3 gm. of sodium hydroxide for each 100 gm. of blood or tissue the water can be evaporated on a hot plate and the organic matter destroyed at a temperature between 300–400° without appreciable loss of iodine. The char is extracted with water. The solution is dark brown and still contains a

large amount of organic matter. Treatment of the solution of the char with barium hydroxide will remove much of the color, but iodine cannot be determined in this solution by oxidation with bromine, as high results are obtained and the end-point is confused by a pink color. For the destruction of the remaining organic matter a second fusion in a small crucible was found to be necessary. By treatment with barium hydroxide the sodium hydroxide which was originally present and which had been converted into carbonate by the first fusion is regenerated as sodium hydroxide. The excess of barium is removed by just sufficient sulfuric acid, leaving the solution still alkaline with sodium hydroxide. The solution, which has a volume of 400 or 500 cc. after treatment with barium, is evaporated to not more than from 15 to 20 cc., and placed in a small nickel crucible.

The water is driven off in a specially constructed hot air chamber protected from carbon dioxide. The residue in the small crucible is then again fused after adding about 5 gm. more of solid sodium hydroxide. This fusion is identical with that described in the previous article. The solution of the fusion melt is now treated in the same manner as for the determination of iodine previously described, and a quantitative determination of the iodine originally present in 100 gm. of tissue is obtained. It is apparent that the original charring of 100 gm. of tissue can be extracted with water, the sodium hydroxide regenerated with barium hydroxide, and a second 100 gm. of blood or tissue may be added to the filtrate. In this way there is almost no limit to the weight of material which may be treated.

One point of interest will be mentioned which was discovered during the earlier attempts to perfect the method. All traces of iodine may be precipitated from a slightly alkaline solution containing barium by boiling with a small amount of potassium permanganate and a few drops of alcohol. The iodine is carried down in the barium manganese precipitate, probably in the form of barium iodate, leaving the solution practically free from manganese, and entirely free from iodine. This fact, however, could not be utilized for the determination of iodine, as it was found extremely difficult to get the iodine back into solution from the precipitate. Boiling with sulfuric or sulfurous acid produces only a partial decomposition, much of the iodine remaining with the barium.

The method in detail for the determination of iodine is as follows: 100 cc. of blood, or 100 gm. of muscle or liver,[1] hair, skin, or bone, are placed in a nickel crucible of 500 cc. capacity. 10 cc. of a 30 per cent sodium hydroxide solution are added and the crucible is placed on a hot plate. After all water has evaporated, which requires about 40 minutes, the crucible is placed on a circular metal disc, a little larger in diameter than the bottom of the crucible, over a gas burner and inside a metal cylinder of the dimensions shown in Fig. 1. The flame is adjusted to such a height that all fumes have ceased to be thrown off at the end of not more than 12 minutes. No definite directions can be given for the destruction of the organic matter as each sample of material varies. In general, however, the contents of the crucible form a thick, tarry mass, which rises almost to the top during the first 2 or 3 minutes. The cover cannot be placed on the crucible until after the contents have subsided, which is usually at the end of 6 or 7 minutes. The crucible is covered as soon as possible, as more heat is thereby retained and the destruction accelerated. The best criterion for the finished fusion is the odor and the throwing off of fumes. The heating should be adjusted so that not more than 11 or 12 minutes are required to render the crucible odorless. The char will then have an iridescent black film over the top. It should be very dry and brittle. The crucible is allowed to cool. About 25 cc. of water are added, and the char which sticks very firmly to the crucible is scraped off. The char is ground with a pestle and is boiled in the crucible with about 50 cc. of water. The carbon is removed by filtration on a Buchner funnel, and is then returned to the crucible together with the filter paper and is again boiled with about 30 cc. of water containing 1 to 2 cc. of 50 per cent sulfuric acid. The residue is filtered on a Buchner funnel and the filtrate added to the main portion. To this solution in a beaker 18 gm. of crystalline

[1] When muscle or liver, or other tissue, is used it is essential to make a homogeneous solution in the nickel crucible with sodium hydroxide before the water is all evaporated off. If the material coagulates in large lumps it is sometimes necessary to evaporate down with water on the hot plate two or three times in order to dissolve all the large gelatinous lumps. As soon as complete solution is secured the material can be treated as blood and the water is all evaporated off.

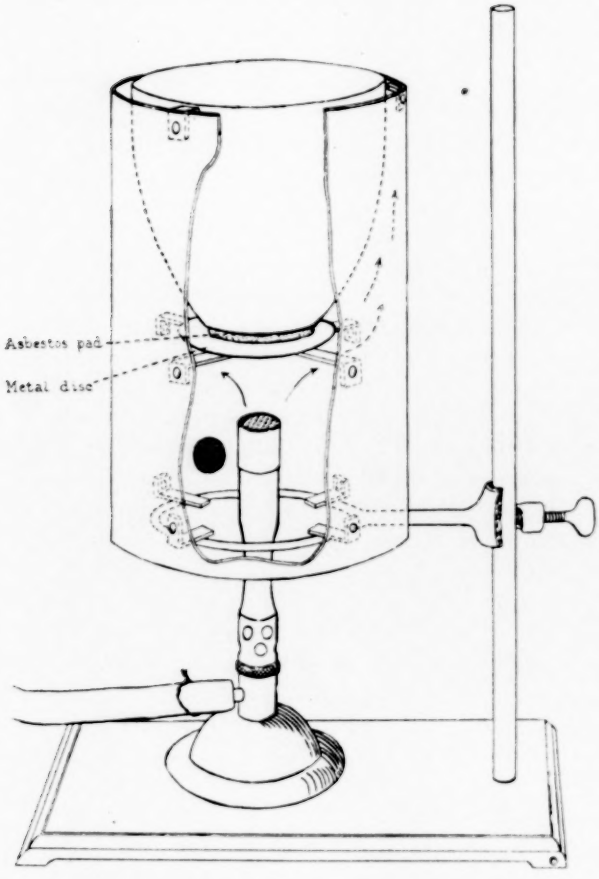

FIG. 1. Cross section of cylinder showing 500 cc. crucible resting on a small asbestos pad supported by a metal disc a trifle larger than the bottom of the crucible. The diameter of the cylinder is 13 cm., of the crucible 10 cm., and of the disc 8.5 cm. The height of the cylinder is 19 cm. A Meker burner 16 cm. high is used.

barium hydroxide[2] are added. The solution is filtered into a suction flask through the Buchner funnel just previously used and still holding the residue of the char. Sufficient sulfuric acid is added to remove all barium, and the solution is again filtered on another Buchner funnel, through a pad of kaolin. The volume at this point is about 400 cc. The solution is placed in a 600 or 800 cc. beaker, a small piece of hard coal is added, and the water is evaporated by rapid boiling on a hot plate. When the solu-

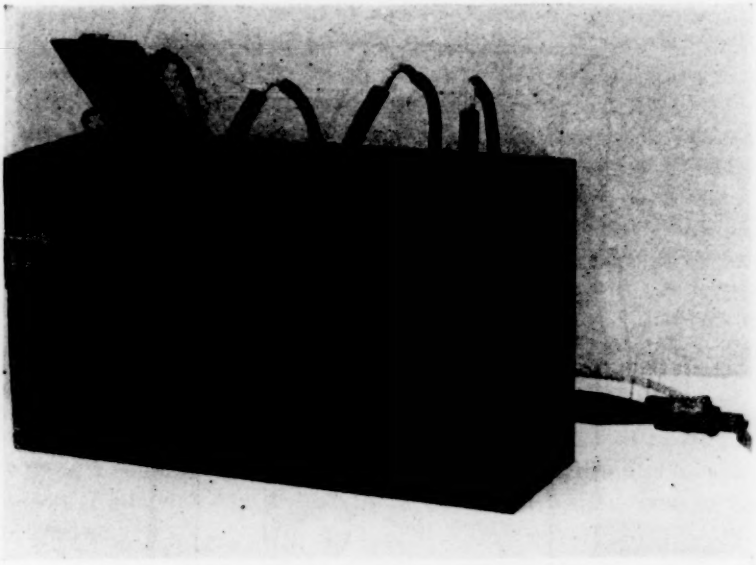

FIG. 2. Hot air oven used to remove water from the small crucible and at the same time protect the sodium hydroxide from carbon dioxide.

[2] The barium hydroxide is prepared from technical commercial barium hydroxide which is twice purified by solution in boiling hot water, filtration through a Buchner funnel, and crystallization of the barium hydroxide from the filtrate by cooling to 15 or 20°. The barium hydroxide should be tested for iodine by precipitating about 100 gm. of the material with carbon dioxide from an aqueous solution after the addition of about 3 gm. of sodium hydroxide. The barium carbonate is removed by filtration, the sodium carbonate in the filtrate is neutralized with phosphoric acid, and the regular method for the determination of iodine carried out.

tion has been reduced to 15 or 20 cc. it is transferred to a nickel crucible of 100 cc. capacity. All but the last trace of water is removed by heating in the hot air chamber (Figs. 2 and 3). The fusion is then carried out, as described in the preceding

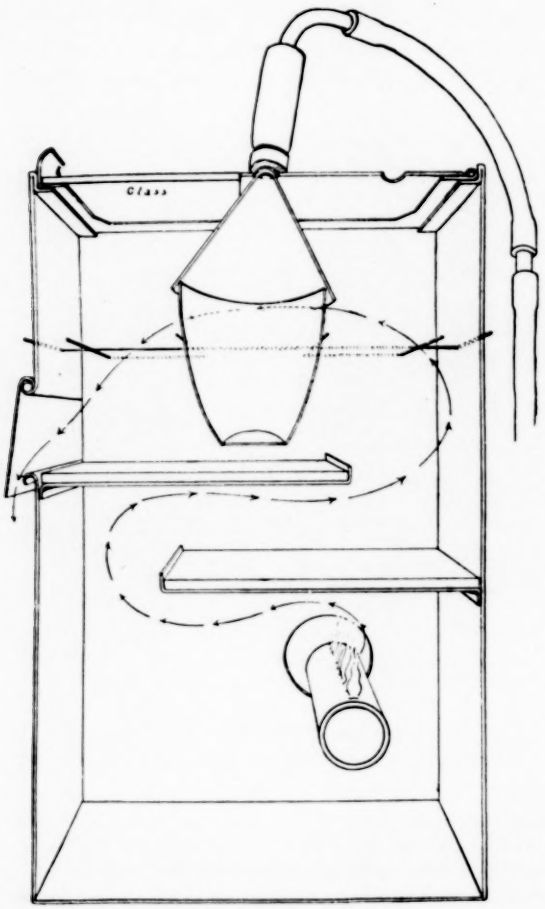

FIG. 3. Cross section of oven showing crucible supported by wires. The temperature of the oven is 180°C. A rapid current of air is blown over the surface of the crucible by means of the inverted funnel.

article. In order to minimize the possibility of securing high results, it is very important to carry out the second fusion, using as little nitrate as possible. The small crucible contains a large percentage of carbonate, which forms during the evaporation of the water, so that the best conditions for a satisfactory fusion are obtained by adding about 4 or 5 gm. of solid sodium hydroxide just prior to the fusion. The organic matter should then be allowed to decompose without the aid of any nitrate until all active liberation of gas has ceased. The entire amount of nitrate added can then be reduced to a few milligrams. Not more than 4 or 5 mg. should be added at any one time. The temperature should be such that the fusion proceeds rapidly, but not so high as to cause the sodium hydroxide to creep up the sides of the crucible. The solution of the fusion melt must be perfectly clear. If it is the least turbid it should be filtered on a suction funnel through a layer of kaolin. Methyl orange is added to the solution which is then acidified and the iodine determined as described in the preceding paper. It is of the highest importance to make frequent tests on all the solutions used. A perfect blank should be secured which will stay colorless at least 15 minutes after the addition of starch and potassium iodide. The Buchner funnels and flasks should not be used for any other work. The filter paper and all utensils should be tested for the presence of the minutest amounts of iodine. The work should be carried out in a room in which iodine is not used and care must be exercised to prevent contamination by potassium iodide used in the titration.

Accuracy of Results.

In order to show the accuracy of results after charring 100 gm. of the material and determining the iodine as described, the method was carried out with blood. 100 cc. of blood were charred in a 500 cc. crucible and the water extract was treated with barium hydroxide, evaporated to small volume, and the residue again fused in a small crucible. Iodine was then determined in the water solution of the fused material. The results obtained are given in Table I.

In order to show that added iodine can be determined by this method in the presence of the products from 100 cc. of blood, iodine as potassium iodide was added to the water solution after charring and fusing 100 cc. of blood (Table II). The method was then carried out as described.

TABLE I.

Experiment No.	Iodine content of 100 cc. of blood.
	mg.
1	0.013
2	0.014
3	0.015
4	0.016
5	0.017
6	0.016
7	0.010
8	0.013
9	0.013
10	0.013
11	0.014
12	0.011
13	0.010
14	0.011
15	0.010
16	0.010
17	0.011
18	0.014
19	0.011
20	0.012
21	0.016
22	0.014
23	0.013
Average.....	0.013

The difference between the amount added and the amount found averaged 0.013 mg. of the iodine, which is the average iodine content found for 100 cc. of blood.

The iodine was then added as potassium iodide to the blood before it was charred and all steps of the method were carried out as described. The results obtained are given in Table III. The total amount of iodine present is the sum of the added iodine

and 0.013 mg. of iodine, which is the average amount found in 100 cc. of blood.

The difference between the total iodine present and the amount found in Table III averages about 2.5 to 3 per cent of the total amount present. When the iodine is added after the blood has been charred, there is no loss of iodine due to any of the steps in the method. The only actual loss of iodine occurs during the charring of the blood. By adding different amounts of iodine to the blood it has been shown that this is not an absolute loss,

TABLE II.

Iodine added.	Iodine found.	Difference.
mg.	*mg.*	*mg.*
1.003	1.016	0.013
1.003	1.015	0.012
1.003	1.017	0.014
1.003	1.012	0.009
1.007	1.028	0.021
1.002	1.017	0.015
1.002	1.017	0.015
1.002	1.017	0.015
1.002	1.017	0.015
1.002	1.017	0.015
1.007	1.011	0.004
1.007	1.011	0.004
1.001	1.025	0.024
1.001	1.012	0.011
1.001	1.018	0.017
1.001	1.005	0.004
Average		0.013

but a percentage loss. The same percentage loss occurred when 1, 0.5, 0.3, or 0.1 mg. was used, and, since no greater loss could occur under the conditions for the iodine determined in 100 cc. of blood without the addition of more iodine, the results may be regarded as quantitative except for the same percentage loss, that is, no more than 3 per cent. Although this is a serious loss for a quantitative method, in this particular case the total amount is so small that the 3 per cent loss due to the first step in the method is actually less than can be read on the burette with

TABLE III.

Total amount of iodine in 100 cc. blood.	Iodine found.	Difference.	Average loss.
mg.	*mg.*	*mg.*	*per cent of total iodine*
1.021	1.000	−0.021	
1.018	0.990	−0.028	
1.018	0.995	−0.023	
1.021	1.006	−0.015	
1.016	1.000	−0.016	
1.013	1.000	−0.013	
1.018	0.984	−0.034	
1.018	0.978	−0.040	
1.018	0.980	−0.038	
1.018	0.989	−0.029	
1.018	0.993	−0.025	
1.018	0.976	−0.042	
1.018	1.002	−0.016	
1.018	0.992	−0.026	
1.019	0.984	−0.035	
1.019	0.999	−0.020	
1.016	0.988	−0.028	
1.016	1.011	−0.005	
1.016	0.989	−0.027	2.5
0.514	0.508	−0.006	
0.513	0.495	−0.018	
0.515	0.493	−0.022	
0.516	0.509	−0.007	
0.516	0.513	−0.003	2.2
0.313	0.299	−0.014	
0.312	0.299	−0.013	
0.316	0.312	−0.004	
0.316	0.306	−0.010	
0.312	0.307	−0.005	2.9
0.115	0.111	−0.004	
0.115	0.116	−0.001	
0.115	0.111	−0.004	
0.115	0.107	−0.008	
0.115	0.111	−0.004	
0.118	0.116	−0.002	3.0

accuracy. For normal blood the amount would be 0.0004 mg. of iodine or 0.004 cc. of 0.005 N thiosulfate.

FORMATION OF THE GUM, LEVAN, BY MOLD SPORES.*

I. IDENTIFICATION AND QUANTITATIVE DETERMINATION.
II. MODE OF FORMATION AND INFLUENCE OF REACTION.

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I. Identification and Quantitative Determination.

The formation of the gum, levan, during the bacterial decomposition of sugar has been noted by Greig-Smith and Steel (1) and Owen (2). The former and Taggart (3) agree in the identification of chemical and physical properties of this bacterial gum called levan. In a consideration of the microbiological agencies responsible for sugar deterioration we have found that molds are by far the most dangerous single group of microorganisms normally occurring in sugars of all descriptions (4). However, in establishing the fact that mold spores contain enzymes and can therefore cause the inversion of sucrose without germination or growth, it is also noteworthy to observe that mold spores contain an enzyme which can produce gum in sterile sugar solutions (5) of 10 and 20 per cent concentration. In order to identify this gum the following procedure was employed.

Identification of Levan.

A pure pedigreed culture of *Aspergillus sydowi* Bainier was used to seed a large number of Petri dishes containing Kopeloff's agar (4). After the spores had developed in profusion they were floated off the plates with sterile distilled water onto a sterile filter paper (Whatman No. 4) and washed thoroughly (about 100

* Read at the St. Louis meeting of the Sugar Division of the American Chemical Society, April 15, 1920.

cc. of sterile distilled water per plate being used). The mold spores were washed into a sterile flask, 10 per cent of chloroform was added, and the suspension heated to 62.5°C. for 1 hour. The suspension was thoroughly ground with sterile sand and then added to 500 cc. portions of sterile 10 per cent sugar solution and incubated at 40°C. for 10 days. The solutions were shaken frequently and found to be sterile on plating out at the end of this period. 10 cc. of the sugar solution from control flasks uninoculated, when made up to 50 cc. volume, gave a single polarization of 13.2°V, while 10 cc. of the gum solution similarly diluted with alcohol gave a single polarization of 5.3.

The supernatant gum solution was then siphoned from each flask without disturbing the spore residue, which was discarded. Five volumes of 95 per cent ethyl alcohol were added to the gum solution which had been made alkaline with NaOH, the solutions being shaken thoroughly at frequent intervals and permitted to stand over night at room temperature. The alcoholic super-

TABLE I.

Chemical Composition of Levan Produced by Mold Spores.

Rotation.	Moisture.	Ash.	Hydrolysis.	Melting point.
	per cent	*per cent*		°C.
−40.9°	0.17	0.16	−87.3 to −90.0	200

natant liquid was removed and the precipitated gum thoroughly washed with successive portions of alcohol. The precipitate was then dissolved in the least possible amount of cold sterile distilled water and shaken thoroughly in a shaking machine for 4 hours. Again the gum was precipitated by alcohol in the manner described above and the process repeated several times until a white flocculent precipitate was obtained. This was partially dried on filter paper and finally transferred to an unglazed porcelain plate where it remained for 18 hours at 20°C. The dry gum was then powdered and used for the determinations described below.

The specific rotation of this gum was about −40° in a 0.25 per cent solution, as may be seen in Table I.

The moisture content was 0.17 per cent, and ash 0.16 per cent. After hydrolysis with concentrated hydrochloric acid the specific

rotation was about −87 to −90°, which coincides with the results obtained by Greig-Smith and Steel (1) and Taggart (3) and indicates that the gum hydrolyzes quantitatively into levulose. The melting point was not sharply defined but occurred at about 200°C., when the gum coalesced and puffed, rising sharply in the capillary tube.

TABLE II.

Effect of Reagents on Levan.

Treatment.	Result.
Oxidation with concentrated nitric acid, boiling	Oxalic acid.
Cold water	Soluble.
Hot water solution after cooling	Mucilaginous.
Prolonged heating at 100°	Unchanged.
Acid hydrolysis	Levulose.
Baryta	Precipitate.
Lime-water	No precipitate.
Milk of lime	Precipitate.
Strontium	"
Lead subacetate	"
Ammonium lead acetate	"
Litharge, cold	Not dissolved.
Fehling's solution	" reduced.
Ammoniacal mercuric nitrate	" "
Iodine	No blue.
Cuprammonia	Dissolves (does not deposit on standing).
Copper sulfate	Precipitate.
Ferric chloride	No precipitate.
Tannic acid	" "
Acid nitrate mercuric	" "

The dry gum is light yellowish gray giving a blue-gray opaque solution with water. It does not separate from semisolution when allowed to stand for periods of time, or when centrifuged. The effects of various reagents are included in Table II.

With two exceptions, namely precipitation by copper sulfate and lead subacetate, which is probably due to some impurities in the small amount of ash, these results coincide with the same data presented by Greig-Smith and Steel (1) and Taggart (3), and we are therefore led to conclude that the gum under con-

sideration which is formed by the enzymes in mold spores is none other than levan.

Previously we have noted the formation of levan in sugar solutions of varying concentration (5) and the data in Table III are further corroborative of the fact that the enzymes in mold spores causing inversion and levan production are active in highly concentrated sugar solutions, as, for example, 55 per cent in this instance. (All data in this and succeeding tables represent the average of closely agreeing triplicate determinations, unless otherwise stated).

TABLE III.

Formation of Levan in 55 Per Cent Sugar Solutions.

Inoculum.	Single polarization.	Reducing sugars.	Gum.
		per cent	*per cent*
Control	55.1	2.29	
Mold spores	51.7	4.63	1.54

Determination of True Sucrose in the Presence of Gum.

It has been a matter of universal experience that the presence of a gum such as levan makes the polarization of a sugar solution very difficult and, furthermore, renders both single polarization and Clerget liable to errors of considerable magnitude, the former generally giving a lower value and the latter a higher value than that of the sucrose actually present. In making single polarizations of sugar solutions where levan is present, it has been our custom, wherever necessary, to remove the levan by precipitation with alcohol. However, even this method fails to give the true content of sucrose, and therefore it was necessary to develop some more adequate means of making this determination, the details of which follow.

Since the Clerget method involves a minus reading and is based on the use of hydrochloric acid, which has been shown to hydrolyze the gum to levulose, it will readily be seen that the reading will be still further magnified in a negative direction. Therefore, it was necessary to employ a method which would not affect the gum, but would give the true sucrose content. The

invertase method of Hudson (6) was found to fulfill these requirements.[1]

A strong invertase solution was prepared by breaking up 5 pounds of Fleischmann's yeast in a mortar, placing it in a wide mouthed 10 liter bottle with ground glass stopper, adding 125 cc. of C.P. toluene (which is slightly more than the amount recommended by Hudson, while also omitting the addition of water), and keeping it at 20°C. On each day following, a portion of the liquefied mass was filtered and 5 cc. of the extract were added to 95 cc. of a 10 per cent sugar solution. Two drops of glacial acetic acid were added to both the check flasks containing 100 cc. of the sugar solution and the treated solution and held at 30°C. (Both the check flask and that containing 95 cc. of sugar solution as well as the yeast extract were held at 30°C. for 10 to 15 minutes until the contents attained the constant temperature before addition of the yeast extract.) The initial time was noted and polariscope readings were made every minute until one-half of the sucrose was inverted. The 1st day following the addition of toluene to the yeast the extract took 10.5 minutes to reduce the sucrose content one-half; the 2nd day 6.7 minutes were required; the 3rd day 5.6 minutes; the 4th day 4.3 minutes; and the 5th day 4.2 minutes. After the 4th day the bulk of yeast was placed on a large filter paper and the filtrate caught in a sterile flask containing 25 cc. of toluene. By permitting the filtrate to drip into the toluene and keeping the mouth of the receiving flask filled with cotton, the most favorable conditions of asepsis were obtained. Neutral lead acetate was added to the filtrate in a quantity sufficient to insure complete precipitation, and the solution centrifuged and decanted. This was again tested with neutral lead acetate and centrifuged in sterile tubes, the procedure being repeated until no precipitate formed. The very slight excess of neutral lead acetate was removed in a similar manner by the addition of 2 N potassium oxalate, centrifuging, and decanting. The solution was covered with a layer of toluene, then dialyzed in a fish skin membrane against running tap water for 2 days, and finally centrifuged and decanted. The invertase solution thus prepared was covered with a layer of toluene and kept in a sterile, ground glass-stoppered bottle in the refrigerator. It was active enough to invert completely a 10 per cent sugar solution in less than 20 hours at room temperature. In order to compare the efficiency of the invertase solution thus prepared with the Clerget method of hydrochloric acid inversion, several samples of standard granulated (refined) sugar, cane syrup, and 10 per cent sugar solutions were used, the latter with and without the addition of levan. Normal weights (26 gm.) of the materials under consideration were weighed out and made up to 100 cc. For the Clerget determinations 25 cc. of distilled water were added to 50 cc. of solution in a 100 cc. flask,

[1] This procedure was suggested by Assistant Director W. G. Taggart, to whom we are indebted for continued and invaluable assistance.

25 cc. of water added, then 5 cc. of concentrated hydrochloric acid, the solution was heated to 67–69°C. in 3 minutes, held at 69°C. for an additional 7 minutes, then cooled in an ice bath to a temperature several degrees below that of the room, made up to the mark, and polarized at the same temperature as the solutions for single polarization. For the invertase method 5 cc. of invertase were added to 50 cc. of solution made very slightly acid with 10 per cent acetic acid, distilled water was added up to the mark, 1 cc. of toluene added, and the flasks were covered with a watch-crystal and incubated at room temperature for 22 hours.

It will be seen from the data in Table IV that the results obtained for refined sugar by the invertase and Clerget methods agree very closely within the limits of experimental error. Only

TABLE IV.

Comparison of Clerget and Invertase Methods.

Solutions.	Sucrose.		Gum.
	Clerget method.	Invertase method.	
	per cent	*per cent*	*per cent*
1. Refined sugar	10.66	10.67	
2. " "	10.59	10.51	
3. " "	10.59	10.53	
Cane syrup	43.47	43.20	
1.5 per cent levan in 10 per cent sugar solution	11.26	10.17	1.38
0.59 per cent levan in 10 per cent sugar solution	11.00	10.58	0.53
0.33 per cent levan in 10 per cent sugar solution	10.89	10.57	0.40

the averages of closely agreeing duplicate determinations were recorded. As might be expected there is a slight discrepancy in the case of cane syrup. This experiment was repeated with similar results.

Polariscope Determination of Levan.

It is of special interest to note that in addition to applying the invertase method to the determination of sucrose where gum is present, we have here likewise developed a method for the determination of the amount of gum present. In other words, we

have two equations, the one for Clerget or acid inversion, the other for invertase, the difference representing the transformation of gum to levulose. Thus for acid and invertase inversion the following graphic representation may be made of the polariscopic value of a sugar solution containing gum as follows:

Solution contains.	Levorotary.	Dextrorotary.
(*a*) *Acid inversion:*		
Before inversion.....	Levulose + gum.	Sucrose + dextrose.
After "	Levulose + gum as levulose + ½ sucrose to levulose.	½ Sucrose to dextrose + dextrose.
(*b*) *Invertase inversion:*		
Before inversion.....	Levulose + gum.	Sucrose + dextrose.
After "	Levulose + gum + ½ sucrose to levulose.	½ Sucrose to dextrose + dextrose.

Therefore a − b = gum to levulose. Since we know that the specific rotation of levan is −40 and that of *d*-fructose is −92.5, then the difference caused by hydrolysis of the levan is −52.5. Since the specific rotation of sucrose is 66.5, for every per cent of gum in the solution, we have a change in rotation as follows, $\frac{66.5}{52.5} = 1.27$.

Now the difference in sucrose (as may be seen in Table IV) determined by the Clerget and invertase methods for 1.5 per cent levan in a sugar solution is 11.26 − 10.17 or 1.09. Therefore the per cent of gum present is 1.27 × 1.09 or 1.38. This is only 0.12 below the theoretical recovery. Similar results were found where smaller amounts of gum were present.

Thus an adequate method has been established for the quantitative determination of gum, but one which is more reliable for larger rather than smaller amounts of gum in sucrose solution.

SUMMARY.

1. Mold spores (*Aspergillus sydowi* Bainier) contain an enzyme capable of forming gum in sucrose solutions of all concentrations up to the saturation point.

2. The specific rotation of the pure gum was found to be −40, and the melting point about 200°C. Upon hydrolysis levulose was formed.

3. This gum is *levan*, since its chemical and physical properties are identical with those described in the bacterial decomposition of sucrose.

4. The invertase method has been successfully applied in the determination of true sucrose in the presence of gum.

5. Levan may be quantitatively determined polariscopically by using a combination of the Clerget and invertase methods.

II. Mode of Formation and Influence of Reaction.

In Part I we have discussed the identification and quantitative determination of levan. Perhaps the most baffling problem involving the presence of levan in sugar solutions is that concerned with its mode of formation. In an endeavor to throw some light upon this question the following experiments were conducted. 50 cc. of sucrose solutions of varying concentration with and without the addition of C. P. dextrose and levulose were inoculated with 10 cc. of mold spores with and without the addition of 2 cc. of invertase (the latter used to furnish an abundant supply of nascent dextrose and levulose). Sterile distilled water was added to the controls, etc., in such a way as to have all the solutions of equal volume. The flasks, after 1 week's incubation at 45°C., were analyzed for acidity, sucrose by single polarization, reducing sugars (volumetric), and gum by precipitation with alcohol in alkaline solution, and weighing upon tared filter papers.

While there is considerable variation in the results presented in Tables V to VII, nevertheless certain generalizations may be made. In the first place it may be seen in Table V where there were present 34,000 spores per cc. of solution, that there is a direct relation between the amount of sucrose present and the quantity of levan formed; *i.e.*, the largest amount of gum where mold spores are present is 0.19 per cent in 10 per cent sucrose alone. In 5 per cent sucrose the gum drops to 0.13 per cent and in 1 per cent sucrose the gum is only 0.08 per cent. Similarly in Table VI, where there were 47,000 spores per cc. of solution, the more sucrose present the greater is the gum formation. This is

in agreement with Owen's (2) conclusions based on the formation of levan by bacteria. The increase in reducing sugars (dextrose and levulose, both c. p.) bears an inverse relation to the gum formation when only spores are present. On the other hand, when invertase has been added to the spores the largest levan production occurs where there is a greater supply of reducing sugars. This may simply be a corollary to the fact that the greatest amount of reducing sugars occurs where the sucrose content is highest. In this connection it might be well to consider the differences in acidity as measured in terms of 0.1 N NaOH and in Tables VI and IX in pH. While as a rule there is an increase in acidity where invertase has been added to mold spores this does not appear to be of sufficient magnitude to account for the differences obtained. It will be observed that when the polarization of sugar solutions inoculated with invertase and mold spores is compared with invertase alone almost invariably the latter is higher than the former, and this despite the fact that an inoculation of mold spores causes a reduction in polarizations when compared with the controls, because some of the nascent reducing sugars from sucrose are used for gum formation. An explanation may be found in the fact that the true acidity of the solutions containing invertase is greater than where mold spores have been added, and similarly the true acidity of the control solutions was generally higher than where mold spores had been added. This was obscured in the method of titrating 15 cc. of the solution with 0.1 N NaOH using phenolphthalein as an indicator, for in such cases the apparent acidity indicated was greater in the case of an inoculation with mold spores than in the controls. However, by adopting the methods and procedure outlined by Clark and Lubs (7), in their notable application of physical chemistry to bacteriology, it was found that the pH values obtained made certain important facts evident; namely, that the pH values of the mold spore inoculation indicated the presence of less true acidity than was to be found in the controls. Consequently it is to be expected that the presence of mold spores would tend to reduce the acidity present where invertase was used. This condition also accounts for the fact that in the solutions containing invertase and mold spores there was less gum formed than where mold spores alone were used, and gum formation is

best aided by the absence of acidity. It would be of interest in this connection to compare spore suspensions of many fungi with a view to ascertaining the pH values, and this study will be carried out in the near future.

TABLE V.

Formation of Levan by Mold Spores in Sugar Solutions of Varying Concentrations.

Inoculum.	Single polarization.	Reducing sugars.	Gum.	Titratable acidity of 5 cc.
Solution A. 10 per cent sucrose.				
		per cent	*per cent*	*cc. 0.1 N NaOH*
Control	7.5	1.26	0	0.02
Mold spores	6.5	1.47	0.19	0.02
Invertase	−2.8	10.00	0	0.10
Spores and invertase	−1.1	10.00	0.06	0.09
Solution B. 5 per cent sucrose + 2.5 per cent each of dextrose and levulose.				
Control	2.4	5.00	0	0.07
Mold spores	2.7	5.88	0.13	0.07
Invertase	−2.5	9.52	0	0.19
Spores and invertase	−1.0	8.69	0.04	0.16
Solution C. 1 per cent sucrose + 4.5 per cent each of dextrose and levulose.				
Control	−1.6	8.33	0	0.13
Mold spores	−1.7	8.69	0.08	0.13
Invertase	−2.3	8.69	0	0.30
Spores and invertase	−1.1	7.57	0.04	0.16
Solution D. 5 per cent dextrose and 5 per cent levulose.				
Control	−2.0	8.69	0	0.17
Mold spores	−2.1	8.69	0.12	0.20
Invertase	−2.0	8.69	0	0
Spores and invertase	−0.6	7.81	0.11	0.14

It will be seen from Tables V to VII that in the absence of sucrose in solutions containing 5 per cent each of C. P. dextrose and levulose there has been as much gum formed as in solutions containing 1 per cent of sucrose and 4.5 per cent each of dextrose

and levulose. These data contradict those of previous investigators in that no formation of gum has heretofore been recorded where C. P. dextrose and levulose have been used without the addition of sucrose. In order to test these Merck chemicals for

TABLE VI.

Formation of Levan by Mold Spores in Sugar Solutions of Varying Concentrations.

Inoculum.	Single polarization.	Loss, single polarization.	Reducing sugars.	Gain, reducing sugars.	Gum.	Titratable acidity of 15 cc.	Reaction.
Solution A.							
			per cent	*per cent*	*per cent*	*cc. 0.5 N NaOH*	*pH*
Control	8.0		0.17		0	0.30	7.1
Mold spores	5.9	2.1	2.14	1.97	0.16	0.06	7.0
Invertase	−2.2	10.2	8.47	8.30	0	0.35	5.1
Spores and invertase	−0.8	8.8	8.58	8.41	0.08	0.32	6.7
Solution B.							
Control	2.4		4.43		0	0.28	4.7
Mold spores	1.8	0.6	5.77	1.34	0.08	0.42	6.7
Invertase	−0.5	2.9	8.33	3.90	0	0.45	4.9
Spores and invertase	0.6	1.8	7.69	3.26	*	0.34	6.5
Solution C.							
Control	−0.9		7.32		0	0.40	4.6
Mold spores	−0.2		7.51	0.19	*	0.62	6.2
Invertase	−1.6	0.7	8.33	1.01	0	0.30	6.2
Spores and invertase	−0.5	0.4	7.69	0.37	*	0.48	6.3
Solution D.							
Control	−1.8		7.69		0	0.70	4.7
Mold spores	−0.5		7.69		*	0.64	6.1
Invertase	−1.8		7.69		0	0.70	5.0
Spores and invertase	−0.5		7.32		0.01	0.61	6.2

* Negligible amounts.

the presence of sucrose, normal weights of a 10 per cent solution were taken for single polarization and Clerget at room temperature (25–30°C.) for 20 hours. The solutions were brought to neutrality with strong NaOH and acidified with two drops of

10 per cent acetic acid before making up to the mark. The results obtained proved that these sugars were entirely free from sucrose which might influence gum formation. Such evidence would seem to indicate that the formation of gum from sucrose requires the intermediate formation of reducing sugars, whether in the nascent state or otherwise. A further discussion of this conclusion will be considered in the experiments which follow, concerning the reaction of the medium.

TABLE VII.

Formation of Levan by Mold Spores in Sugar Solutions of Varying Concentrations.

Inoculum.	1 per cent sucrose + 4.5 per cent dextrose + 4.5 per cent levulose.				5 per cent dextrose + 5 per cent levulose.			
	Single polarization.	Loss, single polarization.	Reducing sugars.	Gum.	Single polarization.	Loss, single polarization.	Reducing sugars.	Gum.
			per cent	*per cent*			*per cent*	*per cent*
Control	−1.4		8.69	0	−2.0		8.96	0
Mold spores	−0.3	−1.1	8.82	0.07	−0.5	−1.5	9.09	0.06
Spores and invertase	−0.5	−0.9	8.89	0.06	−0.5	−1.5	8.96	0.06

Influence of Reaction on the Formation of Gum.

In order to approach the study of the formation of levan from still another angle, sucrose solutions with and without the addition of invertase were treated with varying amounts of alkali. Since an excess of hydroxyl ions will prevent the enzyme, invertase, from being active, it was hoped that a point of alkalinity might be obtained where the invertase would be inactivated but the levanase would perhaps function in the formation of gum, so that the fact could be established whether or not invert sugar is necessary for the formation of levan. Sterile 0.1 N NaOH was first added at the rate of 2, 3, and 4 cc. to 50 cc. of 10 per cent sugar solution, untreated, inoculated with mold spores plus 2 cc. of invertase.

The results of 1 week's incubation at 43°C., are presented in Table VIII from which it appears evident that with 2 cc. of 0.1 N alkali there is practically no difference in the per cent of gum

formed whether invertase is present or absent, while with 3 cc. of alkali there is a marked increase in gum formation in the presence of invertase. A further increase in alkalinity, namely 4 cc., gives similar results in lesser amount. These data indicate that the reaction of the medium is a limiting factor in the formation of levan from sucrose by mold spores, in so far as it influences invertase activity. It is quite evident that this activity is extremely sensitive to slight changes in reaction and becomes inhibited by any appreciable concentration of acidity or alkalinity.

TABLE VIII.

Formation of Levan by Mold Spores in Sugar Solutions Receiving Alkali.

Inoculum.	Titratable acidity of 5 cc.	Single polarization.	Reducing sugars.	Gum.
Addition 2 cc. of 0.1 N NaOH.				
	cc. 0.1 N NaOH		*per cent*	*per cent*
Control	0.02	6.4	1.49	0
Mold spores	0.02	5.9	1.92	0.08
Spores and invertase	0.05	−0.5	8.33	0.09
Addition 3 cc. of 0.1 N NaOH.				
Control	0.01	6.4	1.09	0
Mold spores	0.01	6.0	1.79	0.09
Spores and invertase	0.03	4.5	3.03	0.22
Addition 4 cc. of 0.1 N NaOH.				
Control	0.02	6.4	1.11	0
Mold spores	0.01	5.3	1.12	0.07
Spores and invertase	0.03	4.4	3.23	0.19

The use of invertase in the above solutions was to insure an adequate source of *nascent* dextrose and levulose by the means of an agent which would not otherwise affect the biological processes involved. Naturally, there was always present an adequate supply of reducing sugars. Since the maximum gum formation occurs in the presence of invertase (with 3 cc. of alkali) and where there is actually a smaller amount of reducing sugars formed, it is apparent that the most favorable source of gum formation is the nascent dextrose and levulose formed from sucrose by invertase.

In Table IX similar data are presented employing a wider range of alkalinity varying from 0.3 to 2.0 cc. of N NaOH per 50 cc. of 10 per cent sucrose solution. While these results are quite variable due to a light inoculation of 24,000 spores per cc. of solution, nevertheless there again seems to be evidence to the effect that gum formation goes forward most favorably in the presence of invertase which is responsible for the presence of nascent dextrose and levulose. However, several of these solutions were contaminated with bacteria, which were probably

TABLE IX.

Effect of Alkalinity on Formation of Levan by Mold Spores.

N alkali.	Control.			Mold spores.				Mold spores and invertase.			
	Single polarization.	Reducing sugars.	pH	Single polarization.	Reducing sugars.	Gum.	pH	Single polarization.	Reducing sugars.	Gum.	pH
cc.											
0.3	8.1	0.15	7.3	8.1	1.67	0.2	7.3	−0.5	8.33	0.39†	7.0
0.4	9.0	0.16	6.8	8.8	0.49	*	7.3	1.0	7.18	0.46†	6.7
0.5	8.8	0.18	7.5	8.8	0.36	0.5	7.9	0	8.46	0.09	7.1
0.6	8.8	0.20	7.1	9.1	0.31	*	7.6	0.6	8.11	0.34	7.1
0.7	8.3	0.17	7.3	8.8	0.20	*	7.5	0.8	7.03	0.22†	6.6
0.8	8.3	0.08	7.6	8.3	0.18	0.2	7.8	2.0	6.06	0.58†	6.9
1.0	8.3	0.10	7.9	8.9	0.06	*	8.1	1.2	7.14	0	7.1
1.5	8.4	0.07	8.1	8.5	0.10	*	8.3	8.4	0.01	0.01	7.6
2.0	8.3	0.05	8.3	8.6	0.06	0.04	8.3	8.6	0.06	0	7.3

* Negligible amount.

† Bacterial contamination.

responsible for some increase in gum formation. A point of considerable interest in this experiment is that the pH values of the solutions containing mold spores were higher than the control flasks, showing again that the reaction of that inoculum tends to make the reaction of one medium more alkaline, while the addition of invertase invariably increases the acidity. It will be observed that the maximum gum formation occurred with the addition of 5 cc. of 0.1 N alkali or at a pH value of 7.1 which is practically true neutrality (7.0 representing neutrality). Thus we are led to believe that the maximum gum formation occurs

where nascent dextrose and levulose are available and the reaction of the medium is practically neutral.

The influence of marked changes in alkalinity of the medium is shown in Table X, where there were 108,000 spores per cc. of solution where from 0.6 to 6.0 cc. of N NaOH have been added to 70 cc. of 10 per cent sucrose solution. Here again the maximum gum formation occurs in the presence of nascent dextrose and levulose (invertase added) where the reaction is nearly neutral. In fact three times as much gum is formed as where invertase has not been added. An increase in alkalinity causes an inhibition in invertase activity (as seen by the amount of reducing sugars present), and also a reduction in gum formation.

TABLE X.

Effect of Alkalinity on Formation of Levan by Mold Spores.

N alkali.	Control.			Mold spores.			Spores and invertase.		
	Single polarization.	Reducing sugars.	Gum.	Single polarization.	Reducing sugars.	Gum.	Single polarization.	Reducing sugars.	Gum.
cc.		*per cent*	*per cent*		*per cent*	*per cent*		*per cent*	*per cent*
0.6	9.0	0.22	0	8.5	0.12	0.10	6.5	2.67	0.30
2.4	8.7	0.14	0	8.8	0.20	0.04	8.7	0.23	0.09
3.6	8.5	0.20	0	8.7	0.15	0.04	8.6	0.14	0.01
6.0	8.1	0.13	0	8.4	0.06	0	8.2	0.07	0

At the greatest concentration of alkalinity there were present sufficient hydroxyl ions to stop entirely invertase activity which is paralleled by the absence of gum formation, thus indicating the causal relation previously referred to.

These experiments were repeated twice, the results confirming those set forth above, but it is superfluous to include these data at this point.

In the light of the foregoing experimentation we are able to arrive at a satisfactory explanation of the phenomenon of gum formation in sterile sugar solutions inoculated with certain microorganisms. Namely, the formation of gum from sucrose depends upon the inversion of the latter. The most favorable conditions are those where nascent dextrose and levulose are present in abundance when the reaction is optimum or practically neutral (in terms of hydrogen ion concentration). We have arrived

through somewhat different considerations at the same conclusion, in this respect, as that of Greig-Smith. There is, moreover, good reason to believe that nascent levulose is even a more favorable source for gum formation than nascent dextrose and that the reaction between levan and levulose (which is formed by hydrolysis) may be, to some extent, a reversible one. The basis for this hypothesis may be found in Table VII, where it is seen that there is an increase in polarization where nascent dextrose and levulose are being used as a source of gum formation, for the polarization of the controls is -2.0, while that of spores and invertase is only -0.5, indicating that more levulose than dextrose has been utilized. It follows that as a secondary source of supply an increase in sucrose involves an increase of nascent dextrose and levulose. Consequently it is justifiable to state that gum formation likewise depends on sucrose. Furthermore, we have evidence to show that, in the absence of sucrose or nascent dextrose and levulose, gum may be formed in small amounts from solutions containing C. P. dextrose and levulose present in equal quantities.

The above facts in no way conflict with the theoretical considerations governing the transformations which carbohydrates may undergo.

SUMMARY.

1. Levan is formed by levanase from mold spores most readily from nascent dextrose and levulose (obtained by the action of invertase on sucrose). There is evidence that nascent levulose is used to a greater extent than nascent dextrose in the formation of levan. In the absence of sucrose and nascent reducing sugars, slight gum formation was obtained with C. P. reducing sugars.

2. Levan is not formed directly from sucrose, but may be formed when the latter undergoes inversion.

3. Appreciable concentrations of acidity or alkalinity inhibit the activity of levanase. The optimum reaction appears to be about pH 7.0.

In conclusion, we wish to express our appreciation for the many helpful suggestions received from Assistant Director W. G. Taggart, Dr. F. W. Zerban, and Mr. W. L. Owen, and for the constructive criticism so generously offered by Dr. C. A. Browne.

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CONTRIBUTION TO THE CHEMISTRY OF PHOSPHOMOLYBDIC ACIDS, PHOSPHOTUNGSTIC ACIDS, AND ALLIED SUBSTANCES.

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INTRODUCTION.

Phosphomolybdic and phosphotungstic acids have long been used in biological chemistry. The ordinary complex acids of commerce are common reagents for proteins, amino-acids, alkaloids, and other organic bases, while the specially prepared solutions of Folin and Denis have in recent years been used for the colorimetric determination of uric acid, adrenalin, phenol, sugar, etc. The chemistry of the complex acids is, however, little understood.

Having had frequent occasion to use the complex acids both as protein precipitants and as color reagents, the writer was led

to study certain phases of the chemistry of these compounds. With no intention of going into the field of the pure inorganic chemistry of these compounds, attention was confined at first to the color reaction of the Folin-Denis reagents with uric acid, and to the preparation of the ordinary phosphotungstic acid. As the investigation progressed, however, it was found desirable to extend the study so as to cover all the complex phospho-acids of molybdenum and tungsten.

The literature of the complex acids is voluminous and confusing. With the aid of the color reactions which lend themselves to qualitative differentiation as well as to quantitative estimation of the different complex acids, the writer was able to survey the field in a way that was scarcely possible before, and has arrived at a conclusion regarding the present status of our knowledge of the complex acids.

HISTORICAL.

Gmelin discovered that phosphoric acid formed a yellow precipitate in acid solution with ammonium molybdate. The precipitate contained so little phosphorus that he regarded it as unessential. Sonnenschein showed that phosphoric acid was an integral part of the precipitate and suggested its use for the quantitative determination of phosphoric acid. Debray prepared the free phosphomolybdic acid by digesting the yellow precipitate with aqua regia. The ratio of P_2O_5 to MoO_3 in the yellow precipitate and acid was according to Debray as 1:20, and according to Rammelsberg, 1:22. But the unanimous conclusion of Finkener, Pemberton, von der Pfordten, Gibbs (1881–82 *a*), and Hundeshagen that the compound contained 1 P_2O_5 to 24 MoO_3 is now generally accepted.

Scheibler in 1872 described two complex acids of tungsten. He boiled sodium acid tungstate ($Na_6H_8W_7O_{26} + 12\ H_2O$) and half its weight of phosphoric acid, and obtained a crystalline compound of the composition $Na_8H_{11}P_2W_6O_{31} + 13\ H_2O$. From this he prepared the barium compound which on decomposition (with sulfuric acid) gave a free acid $H_{15}PW_{11}O_{43} + 18\ H_2O$. Using a slightly different procedure, he obtained another acid of the composition $H_{11}PW_{10}O_{38} + 8\ H_2O$. He published no analyses, and he assigned the formulas to his compounds with reservation. There is no doubt that the work of Scheibler possesses only historical value.

Later Gibbs (1880–81 *a*) took up the study of the complex acids. He boiled 24 mols of $Na_2WO_4 + 2\ H_2O$ with 2 mols of Na_2HPO_4 and acidified the mixture with nitric acid. The complex acid was precipitated with mercurous nitrate, and the mercurous phosphotungstate was decomposed with hydrochloric acid. The free acid thus obtained contained 1 P_2O_5: 24 WO_3. Sprenger, Kehrmann and Freinkel (1891), and Brandhorst and

Kraut obtained the same compound by slightly different methods and all their analytical results showed that the ratio P_2O_5:WO_3 was as 1:24.

Besides the compounds containing 1 P_2O_5 to 24 MO_3 (M = W or Mo) Gibbs (1880–81 *a*, *b*) reported others with the ratio P_2O_5:MO_3 as 1:22, 1:20, 1:18, and 1:16. These compounds were obtained only in the form of salts. The conditions under which they were formed were not well defined, and he believed that it was almost impossible to predict what complex would be formed under exactly similar conditions. He stated repeatedly that the compounds were "only to be distinguished by analysis," and he also admitted that some of his compounds could not be recrystallized and that consequently their absolute purity could not be guaranteed. Neither was the method of phosphate determination then available entirely adequate for his problem, and among other sources of error need be mentioned only the circumstance that he had to apply at times to his phosphoric acid determination a correction of 8 per cent, a magnitude as large as the difference between the P_2O_5 contents of the 1:24 and 1:22, or between the 1:20 and the 1:18 compounds. In view of all these facts, much of his work is of doubtful significance.

Péchard also reported a number of phosphotungstic acids other than the 1:24 acid. He mixed theoretical quantities of *m*-tungstic acid and phosphoric acid, and claimed to have obtained acids with the ratio P_2O_5:WO_3 as 1:20, 1:16, and 1:12. He did not state his method of analysis, and, as far as can be judged from his published figures, duplicate analyses differing among themselves by 5 per cent or more, there is no convincing evidence that his compounds had the composition he assigned to them.

In 1887 Kehrmann prepared a new phosphotungstic acid by boiling 1 mol of sodium tungstate with 4 mols of phosphoric acid. A set of peculiar circumstances led him to assign to his compound a formula with the ratio P_2O_5:WO_3 as 1:16. As a result of further research, he corrected his mistake a few years later (1892) and concluded that his compound really contained 1 P_2O_5 to 18 WO_3. In collaboration with Böhm (1894 *a*, *b*) he succeeded in isolating the corresponding molybdenum compound from the mother liquor of the barium salt of the 1:24 acid by salting out with NH_4Cl. The ammonium salt crystallized out from the solution as orange-red prisms. The free acid was obtained by treating the potassium salt with theoretical quantity of sulfuric acid and precipitating the potassium sulfate with alcohol and ether. He also observed that the 1:24 phosphomolybdic acid was transformed into the 1:18 acid by free phosphoric acid on long standing, but no method for the preparation of the 1:18 compound was given. (Finkener had previously observed some sodium salt containing 1 P_2O_5 to MoO_3, but, as he did not mention any detail, the discovery of the 1:18 acid is to be attributed to Kehrmann.)

Kehrmann and his collaborators observed that the 1:24 and 1:18 phosphotungstic acids were converted by carbonates into new complexes containing respectively 1 P_2O_5 to 22 WO_3, and 1 P_2O_5 to 17 WO_3. These complex salts corresponded to hypothetical acids of the composition $7\ H_2O{\cdot}P_2O_5{\cdot}22\ WO_3$ and $5\ H_2O{\cdot}P_2O_5{\cdot}17\ WO_3$, but when the salts were boiled with mineral acid the original 1:24 and 1:18 acids were regenerated.

Kehrmann and Freinkel (1892) claimed to have prepared the acid containing 1 P_2O_5 to 21 WO_3 which was formed from the 1:22 salt simultaneously with the regeneration of the 1:24 acid. This finding the writer has not been able to confirm.

Compounds containing much smaller amounts of WO_3 or MoO_3 are also known. Thus the 1 P_2O_5:15 MoO_3 compound was reported by Rammelsberg; the 1 P_2O_5:5 MoO_3 compound by Rammelsberg, Zenker, Debray, Wernke, and Friedheim; the 1 P_2O_5:4 MoO_3 and 1 P_2O_5:2 MoO_3 by Friedheim; the 1 P_2O_5:7 WO_3 by Gibbs (1880–81 *b*) and Kehrmann (1892). All these compounds, if they exist at all, are formed either from the higher acids by decomposition with insufficient alkali or from the molybdate and tungstate with insufficient acid. They have been prepared only in the form of salts, as they are converted by mineral acid into the 1:24 or the 1:18 acid.

The Present Status.

The complex phospho-compounds of molybdenum and tungsten fall into two well defined groups:

Group I.—This group includes the complexes containing 1 P_2O_5 to 24 MO_3 and 1 P_2O_5 to 18 MO_3 (M = W or Mo). They can exist either as free acids or as salts. They are all colored, with the exception of the 1:24 tungsten compound. They are very sensitive to reduction, and precipitable with pyridine in dilute solutions. They are converted by alkali into compounds of Group II.

Group II.—This group includes a number of salts with different P_2O_5: MO_3 ratios. They exist only in the form of salts, being converted by mineral acids into compounds of Group I. They are all white or colorless. They are not sensitive to reduction, and are not precipitable with pyridine in dilute solutions.

Compounds of Group II are not complex *acids*, and they have therefore received little attention in this research.

The properties and reactions which have been found useful in distinguishing the complex acids are shown in Table I.

The terms phosphotungstic acid and phosphomolybdic acid are used loosely to represent different compounds. The ordinary acids have been called phosphoduodecitungstic and phosphoduodecimolybdic acids, and the acids discovered by Kehrmann were named phospholuteotungstic and phospholuteomolybdic acids, indicating the yellow color of these compounds. It seems desirable to have a system of nomenclature of these complex

TABLE I.

Properties and Reactions of Complex Acids.

Reaction.	W		Mo	
	1:18	1:24	1:18	1:24
Color.	Lemon-yellow.	Colorless.	Bright yellow.	Orange.
NH_4 salt.	Soluble.	Insoluble.	Soluble.	Insoluble.
With uric acid.	Blue coloration in alkaline solution.	Brown coloration in acid; blue in NaOH (but not in Na_2CO_3) solution, fading rapidly.	Blue coloration in alkaline solution.	Blue coloration in alkaline or in acid solution with the complex in excess; violet with uric acid in excess.
With SO_2.	Blue coloration growing slowly in acid. Only a trace of color in alkaline solution.	Almost no reaction. Very faint violet coloration.	Blue coloration in alkaline or neutral solution.	Blue coloration in alkaline solution with the complex in excess; lavender-blue with SO_2 in excess.
With ferrous salt.	Blue in alkaline. Almost none in acid solution.	Brown in acid, blue in alkaline solution, fading rapidly.	Blue in alkaline as well as in acid solution.	Blue in alkaline as well as in acid solution with the complex in excess; lavender-blue with the ferrous salt in excess.

compounds based on their composition rather than on their properties. The ratio P_2O_5: MO_3 or simply P_2: M is better suited than the ratio P : M for this purpose, because there are compounds containing 1 P_2O_5 to an odd number of MO_3, in which case the ratio P: M would have a fractional value. Greek numerals might be used, but it is by far simpler to insert an arabic numeral after the word "phospho," the numeral denoting the number of MO_3 to 1 P_2O_5. Thus, the ordinary acid of molybdenum would be phospho-24-molybdic acid, Kehrmann's tungstic acid would be phospho-18-tungstic acid, the salt obtained from this by the action of carbonate would be phospho-17-tungstate, etc.

Conditions of Formation of the Complex Acids.

The formation of the complex acids depends on four factors: (1) acidity or hydrogen ion concentration; (2) concentration of phosphoric acid; (3) concentration of tungstic acid or molybdic acid; and (4) temperature.

Acidity is the most important factor. A definite hydrogen ion concentration is apparently required to condense the simple tungstic acid or molybdic acid into a complex structure which then combines with phosphoric acid. A higher acidity is required to form $(MO_3)_{24}$ than $(MO_3)_{18}$, and for the molybdenum compound than for the corresponding tungsten compound. The concentration of tungstic or molybdic acid and that of phosphoric acid exert their effects in accordance with the mass action, but they are relatively unimportant. Boiling temperature is required to form the complex acids of the 1 : 18 series, but the 1 : 24 acids are formed even at ordinary temperatures.

The acidity of a mixture of sodium molybdate and phosphoric acid is not enough to form any complex acid (Group I). Whatever proportion of the two compounds is used, the solution contains only colorless complex molybdate (Group II), even after long boiling. Addition of hydrochloric acid to an equimolar mixture of Na_2MoO_4 and H_3PO_4 in an amount equivalent to the quantity of Na_2MoO_4 used, produces a mixture of the 1 : 18 and the 1 : 24 acid. The yield in the latter compound increases with increasing acidity.

The acidity of a mixture of 1 mol of $Na_2WO \cdot 2\ H_2O$ with 4 mols of H_3PO_4 suffices to transform all the tungstate into the 1:18 acid, but none of the 1:24 acid is formed. But if hydrochloric acid is added, the 1:24 acid will be formed almost to the exclusion of the 1:18 acid. The colorless complex tungstates are formed only when the solution is alkaline or weakly acid (in the presence of acetate).

Theoretically a mixture of tungstate or molybdate and phosphoric acid contains all the possible complexes, and the equilibrium can be displaced in one direction or another by suitable means. Thus the molybdenum compounds are very susceptible to transformation. A little ammonium chloride added to a solution of phospho-18-molybdic acid will cause the formation of the ordinary yellow precipitate in the course of time at room temperature, and rapidly at higher temperatures. The tungsten compounds are, however, more stable, and a solution of ammonium phospho-18-tungstate may be heated to 60–70°C. without danger of transformation.

Preparation of the Complex Acids.

The methods which have been employed for the preparation of complex acids of the 1:24 series consist in the double decomposition of the mercurous salt by hydrochloric acid, of the barium salt by sulfuric acid, or in the digestion of the ammonium salt with aqua regia. All these methods are at best very tedious. Drechsel suggested the use of ether for extracting the complex acid from an aqueous solution. This procedure simplified the preparation very materially. But the method, whether in Drechsel's original form or as modified by Winterstein, gives not a pure acid but a mixture. The ether extraction, as far as the writer is aware, has been used only for the preparation of the phospho-24-tungstic acid, although Drechsel stated that it could be used for the corresponding molybdenum compound. As a matter of fact, the great solubility in ether is a property common to all complex acids; and they can all be almost quantitatively extracted with ether from strongly acidified solutions.

Kehrmann obtained the phospho-18-tungstic acid by digesting the ammonium salt with aqua regia, and explicit directions were

given. The exact method for the corresponding molybdenum compound was not worked out.

From the study on the formation of the different complex acids and the recognition of their solubility in ether, simple methods have resulted for the preparation of pure complex acids.

Phospho-24-Molybdic Acid.

Dissolve 100 gm. of $Na_2MoO_4 \cdot 2\ H_2O$ in 200 cc. of H_2O. Add 10 cc. of H_3PO_4 (85 per cent) and then 100 cc. of concentrated HCl. Transfer to a 1,000 cc. separatory funnel, add 150 cc. of ether, and shake. Cool under the tap. After standing for 10 to 15 minutes, three layers should be formed. Transfer the lowest layer, which contains nearly all the complex acid, to another funnel. Add 100 cc. of H_2O and shake. Then add 50 cc. of concentrated HCl and some more ether and shake again. Cool. The lowest layer is again transferred to another funnel and washed once more. Transfer the ethereal solution, which should be perfectly clear, to a beaker, add 25 cc. of H_2O and a few drops of concentrated HNO_3,[1] and evaporate on the water bath with occasional stirring until crystals begin to form on the surface. Allow to cool slowly. Yellow octahedra of the composition $3\ H_2O \cdot P_2O_5 \cdot 24\ MoO_3 + 59\ H_2O$ are obtained.

Phospho-18-Molybdic Acid.

Dissolve 100 gm. of $Na_2MoO_4 + 2\ H_2O$ in about 450 cc. of H_2O, add 15 cc. of 85 per cent H_3PO_4 and 80 cc. of concentrated HCl. Boil for 8 hours with return condenser. Cool. Stir in 100 gm. of powdered NH_4Cl. The crystalline precipitate, after settling, is filtered on a Buchner funnel, and sucked as dry as possible. Redissolve the precipitate in an equal weight of water. A part of the precipitate (NH_4-phospho-24-molybdate) remains undissolved. Filter through hardened paper. To the perfectly clear solution add enough solid NH_4Cl to make a 20 per cent. solution. If the solution is not stirred at all, the NH_4-phospho-18-molybdate will replace the NH_4Cl *in situ* and larger crystals are formed in this way than when the solution is stirred. After standing 4 to 8 hours the crystals are transferred to a Buchner funnel and sucked as dry as possible. Redissolve in just enough water and evaporate at a low temperature (40°C.) in a vacuum, until crystals begin to form. (The solution cannot be evaporated with the aid of a free flame or even on the steam bath because the ammonium salt is transformed into the insoluble yellow precipitate of the 1:24 series even at 60–70°C.) Allow to cool slowly to 5–6°C. Transfer the crystals to a Buchner funnel, and suck as dry as possible. The suction is discon-

[1] If the solution turns brown or green in the course of evaporation more HNO_3 should be added.

tinued and the crystals are covered with a liberal quantity of dry ether. Stir to insure mixing, and, after a few minutes, suck as dry as possible. The crystals are then spread on a watch-glass and will be completely dry in an hour or so. The quick drying is necessary, as the product will be contaminated by some yellow precipitate formed from the mother liquor on slow drying. Orange crystals of the composition 3 $(NH_4)_2O \cdot P_2O_5 \cdot 18\ MoO_3 \cdot 11\ H_2O$ are obtained. They should give a perfectly clear solution in water.

The free phospho-18-molybdic acid can be prepared as follows:

Dissolve 50 gm. of the ammonium salt in 100 cc. of H_2O, add 60 cc. of concentrated HCl, and extract with ether in a separatory funnel. Cool. The lowest layer is transferred to another funnel. Add 100 cc. of H_2O and shake; then add 60 cc. of concentrated HCl and some more ether, and shake again. Repeat the washing once more. Transfer the ethereal solution to a tall beaker, add 40 cc. of H_2O, and blow off the ether by means of compressed air. Allow the solution to evaporate over H_2SO_4. (Like the ammonium salt the free 1:18 acid is transformed into the 1:24 acid at high temperatures.) Orange prisms of the composition 3 $(NH_4)_2O \cdot P_2O_5 : 18\ MoO_3 \cdot 11\ H_2O$ are obtained. The solution should give no precipitate with NH_4Cl.

Phospho-24-Tungstic Acid.

This acid is the chief ingredient of the commercial phosphotungstic acid which is never pure. The Kahlbaum and Merck products contain about 10 per cent of the phospho-18-tungstic acid. To prepare pure phospho-24-tungstic acid proceed as follows:

Dissolve 100 gm. of $Na_2WO_4 \cdot 2\ H_2O$ in about 100 cc. of H_2O with the aid of heat. Add 10 cc. of 85 per cent H_3PO_4 and then 80 cc. of concentrated HCl. Allow to cool. The crystalline precipitate which is gradually formed consists of sodium phospho-24-tungstate but is usually contaminated by traces of acid tungstates. After 4 hours or more, filter on a Buchner funnel and suck as dry as possible. Redissolve the precipitate in 120 cc. of H_2O, pour the solution into a liter separatory funnel, add about 70 cc. of ether, and then 40 cc. of concentrated HCl. Shake. After standing a few minutes there should be three layers of liquid. The lowest layer contains nearly all the complex acid. If there are only two layers, more ether should be added and the mixture shaken again. Transfer the lowest layer to another separatory funnel, add about 120 cc. of water and shake vigorously, then some more ether (30 cc.), and finally 40 cc. of concentrated HCl. Shake. After standing, the lowest layer, which should be perfectly clear, is transferred to a beaker. Add 20 cc. of H_2O and

evaporate on a steam bath until crystals begin to form on the surface. Colorless octahedra of the composition 3 $H_2O \cdot P_2O_5 \cdot 24\ WO_3 \cdot 59\ H_2O$ are obtained.

Phospho-18-Tungstic Acids.

While preparing the ammonium salt of the acid in the uric acid reagent of Folin and Denis it was observed that the crystals first deposited had a different form from those which were deposited later. The two kinds of crystals differ also in other properties such as solubility, hardness, etc., but above all they differ in their chromogenic value. When equal weights (10 mg.) of them were dissolved in water, an excess of uric acid (5 mg.) was added, and the solution rendered alkaline with sodium carbonate (5 cc. of 20 per cent solution), the color (in 50 cc.) produced by the form first deposited was about 30 per cent stronger than that produced by the other form. Since analysis later showed that the stronger form contained a smaller per cent of tungsten it must be concluded that they were two different chemical individuals. As they both contain 1 P_2O_5 to 18 WO_3, they are both phospho-18-tungstic acids. For convenience the stronger form will be designated as A, and the weaker form B. The B form has been identified with the leuteophosphotungstic acid of Kehrmann, but the A form is hitherto unknown.[2] The exact relation between the two forms has not been determined, but as there is clearly the possibility of isomerism their properties, preparation, and analysis will be described in detail.

To 200 gm. of $Na_2WO_4 + 2\ H_2O$ dissolved in a liter of water 280 gm. of 85 per cent phosphoric acid were added. The mixture was boiled for 8 hours under return condenser. Toward the end of the boiling the liquid was allowed to concentrate to a volume of about a liter. A few drops of

[2] It must be pointed out that Kehrmann (1887) in his early study of the phosphotungstic acids had observed different crystalline forms of the yellow potassium salt which he prepared. He believed that there were three compounds represented by short prisms (lemon-yellow), monoclinic octahedra (pale yellow), and small needles (yellowish white), and these forms were designated by him as A, B, and C. The C form was deposited first, then followed the other two. However, he studied only the prismatic form A, of which he made some crystallographic measurements, but in his later work he did not refer to these findings.

bromine water were added to decolorize the solution, which turned to pure yellow. After cooling, about 200 gm. of powdered NH_4Cl were stirred in. The crystalline precipitate was filtered by suction, redissolved in water, and reprecipitated with solid NH_4Cl. This process was repeated twice. The precipitate was then dissolved in about 600 cc. of luke warm water (about 50°C.) and the solution[3] allowed to stand in a warm room (37°C.). After a few days the A crystals (2 to 3 mm.) were deposited in characteristic form, unmixed by any of the other kind. When the deposit ceased to grow, the mother liquor was decanted into another beaker. The A crystals were washed with ice-cold water and recrystallized five times from water.

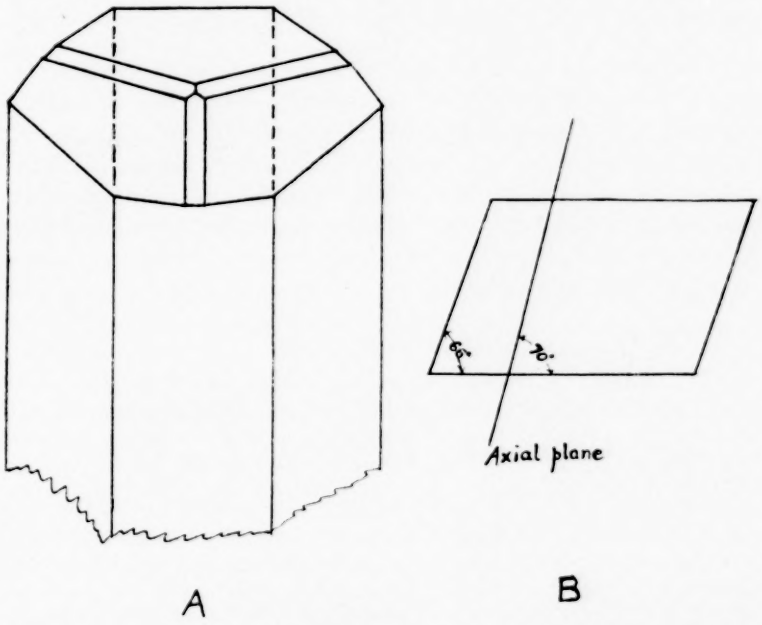

FIG. 1. A. Ammonium A-phospho-18-tungstate. Hexagonal prism truncated by two rhombohedra. The narrow faces are usually not developed.
B. Ammonium B-phospho-18-tungstate. Rhomboidal plate with truncated edges (not shown).

[3] If the solution is somewhat colored, due to reduction of the complex acids by organic dusts, as is usually the case, the crystals of the A form will be much darker than those of the B form. Sometimes the former is almost black, whereas the latter is only faintly green. It is therefore possible to separate the two forms mechanically by means of forceps.

After another week or so the B crystals were formed. When the deposit apparently ceased to grow, it was separated from the mother liquor, dissolved in water, and allowed to recrystallize. The crystals were divided into three crops, the first and last were put aside for other use, while the middle crop was recrystallized four times. Large crystals (1 to 2 cm. long) were obtained.

The preparations were air-dried. Ground samples were used for analysis. Neither preparation gave test for chloride, showing that they were completely freed from the mother liquor which contained a large excess of phosphoric acid.

The yield in the A form was about 30 gm., representing about 20 per cent of the tungstate used.

The method of analysis used embodies certain improvements upon the older procedure. It is at once simple and accurate, and believed to be generally applicable to the analysis of complex phospho-compounds of tungsten and allied elements. The ammonium salt lends itself especially well to analysis, because of the ease with which the non-volatile constituents can be determined.

The method is as follows:

1. Determination of P_2O_5[4].—

The P_2O_5 must be determined in the unignited sample, because some of it is always lost during ignition. 2.5 to 3 gm. of the substance are dissolved in about 25 cc. of warm H_2O, 25 to 30 cc. of a 10 per cent solution of sodium hydroxide are added, and the mixture is heated to boiling until the precipitate which is first formed has entirely redissolved. Continue the heating at a slightly reduced temperature for 20 minutes longer. By this process the complex acid is completely decomposed into phosphate and tungstate. Fusion with Na_2CO_3 as recommended in the literature[5] was never found necessary. After cooling, add 5 to 6 gm. of ammonium chloride. When it has dissolved, add slowly 10 to 15 cc. of magnesia mixture (55 gm. of $MgCl_2 \cdot 6\ H_2O$, 140 gm. of NH_4Cl, and 350

[4] The method for P_2O_5 in phosphomolybdic acids is much simpler. The preliminary boiling with NaOH is not necessary, since the complex molybdenum compounds are decomposed by excess of concentrated ammonia even at room temperature. Incidentally the contamination by silica from glass is practically eliminated and the double precipitation of the magnesium ammonium phosphate may be omitted.

[5] See, for example, Scott, W. W., Standard methods of chemical analysis, New York, 3rd edition, 1918, 482.

cc. of concentrated NH_4OH to the liter), and then one-fourth volume of strong ammonia. After 4 hours the precipitate is filtered and washed with 1:4 ammonia. The precipitate is always contaminated by a considerable amount of silica dissolved from the beaker by the alkali and by a trace of tungstate. To remove these the precipitate is ignited, whereby the silica and tungstic acid are rendered insoluble. If the precipitate is merely redissolved in HCl without ignition, as is recommended in the literature,[5] the impurities are only partly removed. Digest the ignited precipitate with 15 cc. of 2 N HCl for an hour at 80–90°C. with occasional stirring. Filter off the silica on a small filter paper (1½ inches diameter) and wash with ten 5 cc. portions of 0.5 N HCl. Add to the filtrate and washings 5 cc. of magnesia mixture and then very slowly 25 cc. of concentrated ammonia. The precipitate which is now pure magnesium ammonium phosphate is filtered after 4 hours, washed with 1:4 ammonia until free from chloride, ignited with the usual precautions, and weighed. As the amount of phosphate determined is only about 0.1 gm. and the quantity of reagents used is considerable, it is important to make a blank determination and make the correction, if any, accordingly.

2. Determination of WO_3.—Many previous investigators determined the WO_3 in phosphotungstic acids by the difference between the combined oxide ($WO_3 + P_2O_5$) and the phosphoric anhydride. The combined oxide was determined by igniting the ammonium phosphotungstate or the mercurous phosphotungstate obtained by precipitating with mercurous nitrate. It was pointed out by Barber that the ignition residue always weighed less than the theoretical sum of P_2O_5 and WO_3, using compounds of known composition, and he attributed this discrepancy to the partial reduction of WO_3 to some lower oxide. As a matter of fact experiments made by the writer showed that it was not due to this cause, although a trace of tungsten was almost always reduced, imparting a bluish color to the trioxide. When the ignition residue was dissolved in alkali and the P_2O_5 in it determined, the figure obtained was always lower than the true P_2O_5 content of the unignited substance, the difference amounting to 7 to 15 per cent of the P_2O_5 content. It was clear that some P_2O_5 was lost during ignition, and this probably accounted for the discrepancy which Barber had observed.

To determine WO_3 by difference, it is therefore necessary to determine the P_2O_5 in the ignited residue. Accordingly the determination of WO_3 is carried out as follows:

2.5 to 3 gm. of the substance are ignited in a platinum crucible placed inside a porcelain crucible which is heated to bright redness until the residue shows no appreciable loss of weight on successive weighings. The residue is then dissolved in 25 to 30 cc. of a 10 per cent solution of sodium hydroxide and the P_2O_5 in it determined as described above.

3. *Determination of Ammonia and Water.*—The ammonia is determined by distillation and titration in the usual manner, and calculated as $(NH_4)_2O$. The total water is determined by difference.

The results of the analyses representing two preparations each of A and B are shown in Table II and summarized in Table III.

A and B forms must therefore have respectively the formulas

$$3\ (NH_4)_2O \cdot P_2O_5 \cdot 18\ WO_3 + 16\ H_2O$$

and

$$3\ (NH_4)_2O \cdot P_2O_5 \cdot 18\ WO_3 + 11\ H_2O$$

Excepting the number of molecules of H_2O which may be in error by unity,[6] the composition of the compounds as given is beyond any doubt.

The two forms thus differ only in the water content, and the anhydrous forms would have the same composition if the water is all "water of crystallization." There is then the possibility of isomerism and polymerism. But some of the water may be "water of constitution" and an anhydride relation may exist between the two forms.

The free acids of both forms are conveniently prepared from the corresponding ammonium salt by the ether extraction method, although they can also be obtained by digesting the salts with aqua regia.

A-Acid.—

Dissolve 10 gm. of salt in 25 cc. of water. Add 15 cc. of concentrated HCl and an excess (10 cc.) of ether and shake. Transfer the lowest layer to another separatory funnel, add 10 cc. of water, and shake; and then 6 cc. of concentrated HCl and some more ether and shake again. Transfer the ethereal solution to a beaker, add 5 cc. of water, and evaporate on the water bath until crystals begin to form on the surface. The crystallized acid has the composition $P_2O_5 \cdot 18\ WO_3 \cdot 38\ H_2O$.

[6] This is true also for the formulas of other compounds given in this paper.

TABLE II.

Analyses of Ammonium Salts of Phospho-18-Tungstic Acids.

1. P_2O_5

Preparation No.	Form A.			Form B.		
	Weight of sample.	$Mg_2P_2O_7$	P_2O_5	Weight of sample.	$Mg_2P_2O_7$	P_2O_5
	gm.	*gm.*	*per cent*	*gm.*	*gm.*	*per cent*
I	3.0769	0.1431	2.963	3.1564	0.1516	3.067
	2.8336	0.1319	2.972	2.6205	0.1252	3.051
II	3.0538	0.1430	2.990	2.5099	0.1200	3.053
	2.5458	0.1184	2.970	2.7979	0.1339	3.055
Average			2.974			3.056

2. WO_3

	Form A.				Form B.			
	Weight of sample.	Weight of residue.	$Mg_2P_2O_7$ from residue.	WO_3	Weight of sample.	Weight of residue.	$Mg_2P_2O_7$ from residue.	WO_3
	gm.	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*
I	2.4787	2.2353	0.1603	87.58	2.6142	2.4094	0.1125	89.40
II	3.5249	3.1790	0.1420	87.61	3.7840	3.4869	0.1602	89.45
Average				87.595				89.425

3. $(NH_4)_2O$

	Form A.			Form B.		
	Weight of sample.	0.1 N HCl neutralized.	$(NH_4)_2O$	Weight of sample.	0.1 N HCl neutralized.	$(NH_4)_2O$
	gm.	*cc.*	*per cent*	*gm.*	*cc.*	*per cent*
I	1.1320	14.30	3.284	1.1258	14.60	3.370
II	1.2464	15.57	3.248	1.1659	15.12	3.370
Average			3.266			3.370

TABLE III

Composition of Ammonium Phospho-18-Tungstates.

		P_2O_5	WO_3	$(NH_4)_2O$	H_2O (by difference).
Per cent	A	2.974	87.595	3.266	6.165
" "	B	3.055	89.425	3.370	4.150
Ratio	A	1	18.03	2.999	16.35
"	B	1	17.93	3.012	10.72

B-Acid.—

Dissolve 10 gm. of salt in 15 cc. of water. Add 10 cc. of concentrated HCl and extract with 10 cc. of ether. Then proceed as described for the A-acid. The crystallized B-acid has the composition $P_2O_5 \cdot 18\ WO_3 \cdot 40\ H_2O$.

The physical properties of the two forms of ammonium phospho-18-tungstate are shown in Table IV.

TABLE IV.

Properties of Ammonium Phospho-18-Tungstates.

	A	B
Solubility.	51* gm. in 100 gm. H_2O.	122* gm. in 100 gm. H_2O.
Strength.	Tough.	Brittle.
Color.	Lemon-yellow.	Pale yellow.
Deliquescence.	Does not lose water of crystallization easily.	Loses water of crystallization easily and crumbles into powder.
Crystallographic properties.†	Uniaxial. Simple hexagonal prism terminated by two rhombohedra, one of which, probably the unit form, is more strongly developed (Fig. 1). Habit: radial groups consisting of needles elongated in the direction of vertical axis. The second rhombohedron is usually not developed and the crystal then bears a superficial resemblance to a duodecahedron. Optical character, negative.	Biaxial. Probably triclinic. Habit: thin rhomboidal plates with truncated edges (Fig. 1). In one crystal studied the axial plane makes an angle of 70° with one edge and 4° with the other. With the plate lying flat the interference figure shows one axis well within the field. The angle E is estimated as 30°. Optical character, positive.

* Approximately.

† The writer is indebted to Professor C. H. Warren of the Massachusetts Institute of Technology for these observations.

Chemically the two forms are similar. They are both reducible by uric acid in alkaline solution, but the colors produced differ in quality as well as in intensity. For the same weight of WO_3 the A form gives about 30 per cent more color than the B form.

The color of the A form has a tint of green, that of B form, violet. The A form is more sensitive to reduction.

The comparison of the chromogenic values was made as follows:

To 1 cc. of 1 per cent solution of the ammonium salt in a 50 cc. volumetric flask were added, in the order given, 10 cc. of water, 5 cc. of 0.1 per cent uric acid solution (made by dissolving 0.1 gm. of uric acid and 0.1 gm. of lithium carbonate in 100 cc. of H_2O), 0.5 cc. of 5 per cent sodium cyanide, and finally 5 cc. of 20 per cent sodium carbonate solution. The cyanide was used to retard the fading, and it did not affect the relative color values. The color solution was diluted to volume and read in a 50 mm. Duboscq colorimeter. The ratio of the color intensities was

$$\frac{10 \text{ mg. } NH_4 - \text{A-salt}}{10 \text{ mg. } NH_4 - \text{B-salt}} = \frac{25.4}{20} = 1.27$$

or in terms of the anhydrous salts

$$A = 1.3\ B$$

Two 1 cc. portions of the uric acid solution used in the preceding experiment were measured into two 100 cc. flasks. To one were added 10 cc. of 1 per cent solution of the A-salt, and to the other, the B-salt. 10 cc. of 20 per cent sodium carbonate solution were then added to each flask. The color solutions were diluted to volume and read. The ratio of the color intensities was

$$\frac{A}{B} = \frac{25.6}{20} = 1.28$$

TABLE V.

	P_2O_5	WO_3	$(NH_4)_2O$	H_2O (by difference)
	per cent	*per cent*	*per cent*	*per cent*
I	3.100	86.00	5.642	
II	3.095	85.98	5.687	
Average.....	3.098	85.99	5.665	5.247

When treated with sodium carbonate or hydroxide in the cold, the two forms of phospho-18-tungstic acid yield the same decomposition product. This consists of small glistening rectangular plates difficultly soluble in carbonate or hydroxide solution. The ammonium salt obtained by using ammonium carbonate is easily purified by recrystallization from water without undergoing any change. Analysis gave the following results (Table V).

The ratios P_2O_5: WO_3: $(NH_4)_2O$: H_2O in the salt are as 1: 16.99: 4.993 : 13.36. It must therefore have the formula $5\ (NH_4)_2O \cdot P_2O_5 \cdot 17\ WO_3 + 13\ H_2O$.

When the phospho-17-tungstate, whether obtained from the A or the B form, was boiled with hydrochloric acid, the phospho-18-tungstic acid regenerated was exclusively the B form. This was proved by identification of the ammonium salt of the regenerated acid, but it was also easily shown by a simple experiment as follows:

To 1 cc. of 1 per cent solution of the A-salt was added 1 cc. of N Na_2CO_3 solution. After 2 minutes (the decomposition was practically instantaneous) 2 cc. of N HCl solution were added and the solution was heated in the boiling water bath for 30 minutes. A similar experiment on the B-salt was made side by side. 1 cc. of 1 per cent solution of ammonium phospho-17-tungstate and 1 cc. of 1 per cent solution of B-salt were also heated each with 1 cc. of N HCl in the same bath. After cooling, the chromogenic values were compared as described above. The results follow:

10 mg.	A-salt decomposed and regenerated.....	= 9.7 mg.	B-salt
10 "	B-salt " " "	= 9.8 "	"
10 "	colorless salt regenerated.....	= 9.4 "	"

Since 10 mg. of the A-salt are equivalent chromogenically to 12.7 mg. of B-salt, it is clear that by the process of decomposition and regeneration the A-salt has been transformed into the B-salt. Incidentally the experiment showed that regeneration was practically complete.

The exact relation between the two forms of phospho-18-tungstic acid, as well as the more general problem of the structure of the complex acids, stands in need of further study. This is, of course, a problem of inorganic rather than of biological chemistry.

Phosphomolybdictungstic Acids.

Mixed complex acids containing molybdenum and tungsten both of the 1: 18 and of the 1: 24 series have been prepared. The methods of their preparation are very similar to those of compounds containing only one metal. Their properties lie between those of the pure molybdenum and pure tungsten compounds and, to be sure, resemble more the pure compound of the

predominant element. Thus, the soluble ammonium salts of the mixed complex acid of the 1:18 series with high content of molydenum are easily transformed into insoluble salts of the 1:24 series, while those with high tungsten content may be heated to 70°C. without danger of transformation. The sensitiveness to reduction of these compounds runs roughly parallel to their molybdenum content, if one uses as a measure of sensitiveness the intensity of the color produced from a given weight (20 mg.) of the ammonium salt, with an excess of sodium sulfite (10 cc. of 10 per cent solution).

The number of mixed complex acids must be very large, and here the possibility of isomerism is far greater than in the case of the pure molybdenum or tungsten compound. By using sodium tungstate and molybdate in different molecular ratios, the writer has obtained about twenty ammonium salts of the 1:18 series. When these salts are reduced with reagents which do not affect the pure tungsten compound, the amount of color produced is far more than can be accounted for by the amount of molybdenum in the form of pure molybdenum compound in them. Their reduction products also show a great variation in color, whereas the pure molybdenum and pure tungsten compounds give colors which are only slightly different from each other. The color changes from bluish green to violet as the proportion of tungsten is increased. The preparations showed no change by recrystallization from luke warm water. They must therefore be either different chemical individuals or mixtures of two adjacent members of the series; for example, $3H_2O{\cdot}P_2O_5{\cdot}10WO_3{\cdot}8MoO_3$ and $3\ H_2O{\cdot}P_2O_5{\cdot}\ 9\ WO_3{\cdot}\ 9MoO_3$. These compounds would be not only isomorphous but all their properties would be so similar that their separation is practically impossible. A few compounds of the 1:24 series have also been obtained which likewise give different colors on reduction.

The mixed complex acids of the 1:24 series are of no particular interest, and it suffices to mention here that they were prepared from mixtures of tungstate and molybdate by essentially the same methods as described for pure acids of the 1:24 series.

The mixed complex acids of the 1:18 series, on the other hand, count among its members the chief component of the phenol reagent of Folin and Denis, and it is of practical impor-

tance to know the conditions of their formation. These have been found as follows:

1. In the absence of a strong acid (hydrochloric) the complex formed is one with high tungsten content (not very sensitive to reduction), no matter how much molybdate may be used. It appears that under this circumstance the MoO_3 is not sufficiently condensed to form a long chain; the excess MoO_3 remains in the form of colorless compounds of Group II.

2. A strong acid (hydrochloric) must be used in order to incorporate all the molybdic acid used in the complex. The greater the proportion of molybdenum used in the mixture, the larger is the amount of acid required.

3. In the presence of molybdic acid a considerable amount of strong acid may be used without the formation of the complexes of the 1:24 series. However, when too much strong acid is used the 1:24 complexes are formed with corresponding decrease in the yield of the 1:18 complexes.

Since the 1:18 complexes are the desired components of the phenol reagent, the condition should be so adjusted as to obtain the maximum yield of those products. The following method, which gives a solution identical with the "phenol reagent" of Folin and Denis, prepared according to the latest direction, is intended to illustrate the preparation of mixed complex acids of 1:18 series.

Dissolve 100 gm. of $Na_2WO_4 \cdot 2\ H_2O$ and 25 gm. of $Na_2MoO_4 \cdot 2\ H_2O$ in 700 cc. of H_2O. Add 50 cc. of 85 per cent H_3PO_4 and 100 cc. of concentrated HCl. Boil with return condenser for 8 hours. The resulting solution contains mixed acids of the 1:18 series with a bare trace of the acids of the 1:24 series, and may be diluted for use as a phenol reagent.

To prepare the pure 1:18 mixed acid proceed as follows:

Saturate the cooled solution with solid ammonium chloride. Filter the crystalline precipitate on a Buchner funnel. Redissolve in 150 cc. of warm water, and remove the insoluble yellow residue by filtration through hardened filter paper. Add to the clear filtrate an equal volume of concentrated HCl, extract with ether, and proceed as described under the preparation of the phospho-18-molybdic acid. The mixed acids of the 1:18 series suffer transformation at high temperatures to the 1:24 acid, and the solution should be evaporated at room temperature, preferably over H_2SO_4.

If only the ammonium salt is desired, it may be salted out with NH_4Cl from the clear solution and recrystallized from warm water. The free acid should give no precipitate (amorphous) with ammonium chloride, and the ammonium salt should give a perfectly clear solution.

Analysis of the ammonium salt, which was chloride-free, gave the following results (Table VI).

TABLE VI.

	P_2O_5	WO_3	MoO_3	$(NH_4)_2O$	H_2O (by difference).
Per cent.....	3.327	73.57	15.10	3.690	4.313
Ratio.....	1	13.55	4.48	3.060	10.24

The P_2O_5, the combined oxides, and the $(NH_4)_2O$ were determined as described under the phospho-18-tungstic acids; the molybdenum was separated from the tungsten by the tartaric acid method of Rose, and determined directly. It will be noted that the ratio

$$\frac{WO_3 + MoO_3}{P_2O_5} = 18.03$$

showing that the compounds belong to the 1:18 series. The sample analyzed was clearly not a chemical individual, but a mixture of

$$3\ (NH_4)_2O \cdot P_2O_5 \cdot 13\ WO_3 \cdot 5\ MoO_3 \cdot 10\ H_2O$$

and

$$3\ (NH_4)_2O \cdot P_2O_5 \cdot 14\ WO_3 \cdot 4\ MoO_3 \cdot 10\ H_2O$$

It is to be noted also that the ratio MoO_3: WO_3 in the complex is about the same as the ratio of the molybdate to the tungstate used in the preparation, and it seems that individual mixed acids can be obtained by starting with molybdate and tungstate in ratios of integers which give a sum of 18.

Reduction of the Complex Acids.

The phosphotungstic and phosphomolybdic acids are very sensitive to reduction, remarkably so in comparison with the corresponding simple acids. The complex acids are, indeed, akin to chromic acid in their power of oxidation and in their

co'or, and in these respects they, rather than the simple acids, represent molybdenum and tungsten in their periodic relation with chromium.

The complex acids yield on moderate reduction highly colored compounds which resemble the corresponding unreduced compounds in all respects except that of color. Thus, the reduced phospho-24-molybdic and phospho-24-tungstic acids form difficultly soluble ammonium salts. The salts of the reduced phospho-18-molybdic and phospho-18-tungstic acids are soluble, but they can be salted out with ammonium chloride. All are precipitable with pyridine in acid solution and all are extractable with ether. The methods described for the isolation and purification of the unreduced compounds are also applicable to the reduced compounds.

The complex acids arranged in the order of increasing sensitiveness to reduction are as follows:

1. Phospho-24-tungstic acid.
2. B-Phospho-18-tungstic acid.
3. A-Phospho-18-tungstic acid.
4. Phospho-24-molybdic acid.
5. Phospho-18-molybdic acid.

The last two acids react with a variety of mild reducing agents in acid as well as in alkaline solution, while the first three are reduced only when the solution is rendered alkaline. The sensitiveness to reduction runs parallel with the color of the compound, and this parallelism holds also for the mixed complex acids.

The salient feature of the reduction of the complex acids is that the sensitivity is localized in one or two atoms of the metal in the 1:18 series, and probably the same number in the 1:24 series. This view finds support in the fact that complex salts of the 1:17 and 1:22 series formed respectively from the 1:18 and 1:24 acids with the loss of one and two atoms of the metal are totally inert.

Mild reducing agents (ferrous salt, sulfite, uric acid, etc.) affect only the sensitive atoms, and the resulting products are still complex acids. But if the reduction is carried further, for example, by means of an excess of zinc and hydrochloric acid,

the complex structure is disintegrated and all the atoms of the metal originally in the complex can be reduced just as those in the simple acids would be under the same circumstance. The disintegration of the complex is shown by the fact that pyridine no longer produces a precipitate and that after reoxidation the simple tungstic acid or molybdic acid can be seen suspended.

The reduced complex acids are readily restored to the original by oxidation, for example with bromine water. This shows that none of the atoms of the metal is detached from the complex. Consequently the exact composition of the reduced compounds has to be inferred from their oxidation-reduction equivalent, because the difference is too small to be determined with certainty by direct analysis. The oxidation-reduction equivalent was determined by titration of the reduced compound with potassium permanganate and by comparison of the maximum color produced by a given amount of the complex (in the presence of an excess of reducing agent) with that produced by a given amount of the reducing agent (in the presence of an excess of the complex acid). This latter method is applicable, of course, only when the colors produced are the same and are not interfered with by the products of the reaction. The data on the oxidation-reduction equivalents have been obtained mainly from the study of the molybdenum compound.

Just as molybdenum and tungsten trioxides yield on reduction a number of lower oxides, so also do the complex acids give rise to new complexes containing different lower oxides. The exact compounds formed depend on the nature and amount of the reducing agent, and on whether the solution is acid or alkaline. Thus uric acid reduces in acid solution the phospho-24-tungstic acid, forming a brown-colored complex, but if the solution is rendered alkaline by sodium hydroxide the compound formed is blue in color. The phospho-24-molybdic acid gives a lavender-blue compound with excess of the reducing agent and a greenish blue compound when the complex acid is in excess. It is of course a matter of common observation that different polyphenols and other substances which react with the "uric acid reagent" and the "phenol reagent" usually produce colors of different shade which cannot be wholly accounted for by the color of the oxidation products of phenols.

No attempt was made in this research to prepare all the possible complexes containing lower oxides of tungsten and molybdenum. But a few of them have been obtained in pure state to indicate the possibility of others.

Phospho-18-molybdic acid is reduced with the loss of 1, 2, or 3 atoms of oxygen. The ammonium salts corresponding to these stages have been obtained pure. The tungsten compounds undergo reoxidation rapidly when removed from the reducing medium. They could doubtless be prepared pure in a non-oxidizing atmosphere, but this experiment has not been undertaken. From their analogy with the molybdenum compounds, however, their composition may be inferred.

Complex compounds formed from the 1:24 acid by the loss of only 2 and 4 atoms of oxygen have been obtained, but the possibility of others is of course not excluded. Here again, the reduced phospho-24-tungstic acids show such a tendency to be reoxidized that one has to be content with drawing conclusions by analogy with the molybdenum compound.

The formulas of the reduced complex acids of the 1:18 series, as inferred from their oxidation-reduction equivalents, may be represented as follows:

	(*a*)	$3\ H_2O \cdot P_2O_5 \cdot 17\ MO_3 \cdot MO_2$
or		$3\ H_2O \cdot P_2O_5 \cdot 16\ MO_3 \cdot M_2O_5$
	(*b*)	$3\ H_2O \cdot P_2O_5 \cdot 16\ MO_3 \cdot 2\ MO_2$
		$3\ H_2O \cdot P_2O_5 \cdot 14\ MO_3 \cdot 2\ M_2O_5$
or		$3\ H_2O \cdot P_2O_5 \cdot 15\ MO_3 \cdot MO_2 \cdot M_2O_5$
	(*c*)	$3\ H_2O \cdot P_2O_5 \cdot 15\ MO_3 \cdot 3\ MO_2$
		$3\ H_2O \cdot P_2O_5 \cdot 12\ MO_3 \cdot 3\ M_2O_5$
		$3\ H_2O \cdot P_2O_5 \cdot 14\ MO_3 \cdot 2\ MO_2 \cdot M_2O_5$
or		$3\ H_2O \cdot P_2O_5 \cdot 13\ MO_3 \cdot MO_2 \cdot 2\ M_2O_5$

It should be noted that it is not a matter of indifference whether, for instance, the compound (*a*) has the first or the second formula. The first contains one tetravalent atom, and the second, two pentavalent atoms of the metal. It is quite conceivable that the molybdenum or tungsten atoms of different valency are responsible for different shades of color of the reduced complex acids as they are in the simple lower oxides. The gradual increase

in the intensity of the blue color obtained in uric acid determinations may be explained by the conversion of $MO_2 \cdot MO_3$ into M_2O_5 in the complex or *vice versa*. It is easy to understand why different shades of color are produced with the same complex acid under different conditions of reduction, especially in the case of the phenol reagent, for here the number of possible structures corresponding to the loss of the same number of oxygen atoms is greatly increased.

Reduction Experiments.

Reduction of Phospho-18-Tungstic Acids by Uric Acid.—Two color solutions representing the maximum color obtainable respectively from 10 mg. of NH_4-A-phospho-18-tungstate and 0.3 mg. of uric acid were prepared as follows: (1) 10 cc. of 1 per cent uric acid solution (made by dissolving 1 gm. of uric acid and 1 gm. of Li_2CO_3 in 100 cc. of H_2O) + 2 cc. of 10 per cent Na_2SO_3 + 1 cc. of 5 per cent NaCN + 1 cc. of 1 per cent NH_4-A-phospho-18-tungstate + 10 cc. of 20 per cent Na_2CO_3, and water to a volume of 50 cc. (2) 1 cc. of 0.03 per cent uric acid solution + 2 cc. of 10 per cent Na_2SO_3 + 1 cc. of 5 per cent NaCN + 10 cc. of 20 per cent Na_2CO_3 + 10 cc. of 1 per cent NH_4-A-phospho-18-tungstate, and water to a volume of 50 cc.

The Na_2SO_3 and NaCN together retarded the fading, but did not intensify the color as NaCN alone would, and in this case unequally. The ratio of the color intensities was

$$\frac{10 \text{ mg. A}}{0.3 \text{ mg. uric acid}} = \frac{22.7}{20}$$

or

$$10 \text{ mg. A} = 0.34 \text{ mg. uric acid}$$

A similar experiment with NH_4-B-phospho-18-tungstate gave

$$10 \text{ mg. B} = 0.32 \text{ mg. uric acid}$$

Since the A form contains 87.6 per cent WO_3, and the B form 89.4 per cent WO_3, it follows that 18.6 WO_3 = 1 uric acid for the A form and 20.2 WO_3 = 1 uric acid for the B form. Considering the inequality of conditions of the color solutions, it may be concluded that 18 WO_3 = 1 uric acid or 3 $H_2O \cdot P_2O_5 \cdot 18\ WO_3$ = 1 uric acid for either form of the complex acid.

Reduction of Phospho-18-Tungstic Acids in Alkaline Solution by Ferrous Sulfate.—1 cc. of freshly prepared 0.005 N solution of ferrous ammonium sulfate in 0.2 N H_2SO_4 was transferred by means of an Ostwald pipette to a 50 cc. volumetric flask. 2 cc. of 5 per cent solution of B-phospho-18-tungstic acid were added, followed by 10 cc. of 20 per cent sodium carbonate solution. 1 cc. of 1 per cent solution of ammonium B-phospho-18-

tungstate was placed in another 50 cc. flask, 5 cc. of the ferrous sulfate solution were added, and diluted with 20 cc. of water, followed immediately by 10 cc. of carbonate solution. Both flasks were then diluted to the mark and mixed. The first solution was not diluted with water before the addition of the carbonate, because the dilute ferrous sulfate solution could not be diluted with water without undergoing considerable oxidation. The dilution was necessary in the second solution to avoid precipitation of the colored compound together with the ferric hydroxide.

The second solution was centrifuged to remove the ferric hydroxide. (Filtration could not be used, because filter paper would absorb some of the blue color.) The solutions were read in a Duboscq colorimeter. The ratio of the color intensities was

$$\frac{\text{1st solution}}{\text{2nd solution}} = \frac{23}{20}$$

Now since preliminary experiments had shown that the colors obtained in the first and second flasks were the maximum colors obtainable respectively from 1 cc. of 0.005 N ferrous salt, and 1 cc. of 1 per cent complex salt, and since the latter contained 89.4 per cent of WO_3, it follows that 1 cc. of 0.005 N ferrous salt $\Rightarrow \frac{23}{20} \times 0.00894 \Rightarrow 0.0103$ gm. WO_3. Whence 8.85 $WO_3 \Rightarrow$ Fe.

A similar experiment with the A form gave

$$\frac{\text{1 cc. of 0.005 ferrous salt}}{\text{1 cc. of 1 per cent complex salt}} = \frac{23.6}{20}$$

Since the ammonium salt of the A-acid contained 87.6 per cent of WO_3, it followed that 1 cc. of 0.005 N ferrous salt $\Rightarrow \frac{23.6}{20} \times 0.00876 \Rightarrow 0.0104$ gm. of WO_3. Whence 8.90 $WO_3 \Rightarrow$ Fe.

In either case the ratio WO_3:Fe was close enough to 9:1 to justify the conclusion that 3 $H_2O \cdot P_2O_5 \cdot 18$ WO_3 loses 1 atom of oxygen when reduced by ferrous salt in alkaline solution.[7]

Comparison of the Colors Obtained by Reduction with Uric Acid and with Ferrous Salt.—1 cc. of freshly prepared 0.01 N solution of ferrous ammonium sulfate in 0.2 N H_2SO_4 was placed in a 100 cc. volumetric flask. In another flask was placed 1 cc. of 0.1 per cent solution (= 1 mg.) of uric acid dissolved with the aid of lithium carbonate. To each flask were then added 2

[7] Sometimes the ferric hydroxide remained in colloidal solution, in which case the experiment was of course discarded. At other times the two color solutions did not match well and slightly different readings may be obtained by different observers. This does not, however, alter the conclusion already reached.

cc. of a 10 per cent solution of B-phospho-18-tungstic acid, followed by 20 cc. of 20 per cent sodium carbonate solution. The solutions were diluted to the mark, mixed, and read in a Duboscq colorimeter. The ratio of the color intensities was

$$\frac{1 \text{ mg. uric acid}}{1 \text{ cc. } 0.01 \text{ N ferrous salt}} = \frac{23.8}{20} = 1.19$$

The experiment was repeated using the A form with the same result. If 1 molecule of uric acid was oxidized by 1 atom of oxygen, the 0.1 per cent solution was exactly 0.0119 N. It is therefore to be concluded that 1 molecule of uric acid reacts with 1 molecule of 3 $H_2O \cdot P_2O_5 \cdot 18\ WO_3$, confirming the results already obtained in the two previous experiments.

When the methods employed in the above experiments were applied to the corresponding molybdenum compound, the results obtained were not rational, probably because different reduction products were formed. This was to be expected from the much greater susceptibility to reduction of the molybdenum as compared with the tungsten compound. But another possible reason was that the molybdenum compound was also more readily decomposed by alkali, so that a part of it may be decomposed instead of being reduced.

Preparation of Ammonium Salts of Reduced Complex Acids: 3 $(NH_4)_2O \cdot P_2O_5 \cdot 17\ MoO_3 \cdot MO_2 + H_2O$.—The phospho-18-molybdic acid is reduced only with the loss of 1 atom of oxygen by ferrous salt in acid solution. The reduction is an incomplete reaction and a large excess of the reducing agent must be used to prepare the reduced compound.

10 gm. of ammonium phospho-18-molybdate and 25 gm. of ferrous sulfate were dissolved together in 60 cc. of 10 per cent sulfuric acid. When all the solid substances had dissolved, 12 gm. of ammonium chloride were stirred in. Immediately a black crystalline precipitate was formed which after standing 10 minutes was filtered on a small Buchner funnel and washed with 20 per cent ammonium chloride solution until free from iron. The precipitate was redissolved in 50 cc. of warm water and evaporated at 60°C. until crystals began to form on the surface and allowed to cool slowly. Black crystals. It lost 12.50 per cent of its weight on gentle ignition. By calculation (on the basis of 1 P_2O_5 to 18 MoO_3 in the residue) 1 gm. of the substance = 0.8295 gm. of MoO_3. 14.30 cc. of 0.01 N permanganate were required to titrate 0.2206 gm. of the substance. Whence 18 MO = O.

The corresponding tungsten compound could be prepared by reduction with slight excess of Zn and hydrochloric acid in the cold, but, as already mentioned, it could not be easily purified.

3 $(NH_4)_2O \cdot P_2O_5 \cdot 16\ MoO_3 \cdot 2\ MoO_2 + H_2O$.—10 gm. of the orange molybdenum salt were dissolved in 50 cc. of H_2O, and 5 cc. of 40 per cent HI and 5 gm. of $NaHSO_3$ were added. The mixture was allowed to stand 24 hours. 10 gm. of solid NH_4Cl were stirred in. After a few minutes the crystalline precipitate was filtered on a Buchner funnel, washed with 20 per cent NH_4Cl solution until free from sulfate, and finally once with ice-cold 5 per cent NH_4Cl solution, and sucked as dry as possible. The product was recrystallized from warm water. Black crystals. It lost 21.45 per cent of its weight on ignition. By calculation 1 gm. of the substance = 0.7447 gm. of MoO_3. 0.2595 gm. of the substance required 14.46 cc. of 0.02 N $KMnO_4$ to discharge the blue color. Whence 18 Mo = 20.

3 $(NH_4)_2O \cdot P_2O_5 \cdot 16\ MoO_3 \cdot M_2O_5 + H_2O$.—10 gm. of the orange salt were dissolved in 50 cc. of H_2O, 5 cc. of 40 per cent HI were added, and a current of H_2S was passed into the solution until the latter was saturated. After 24 hours the solution was filtered to remove the sulfur. The filtrate was treated with 10 gm. of solid ammonium chloride. The crystalline precipitate was filtered and washed with 20 per cent NH_4Cl solution until free from iodide. The product was recrystallized once from warm water. Black crystals, with violet reflex. The preparation lost 18.65 per cent of its weight on ignition. By calculation 1 gm. of the substance = 0.7713 gm. of MoO_3. 22.90 cc. of 0.02 N permanganate were required to titrate 0.2615 gm. of the substance. Whence 18 MoO_3 = 30.

3 $(NH_4)_2O \cdot P_2O_5 \cdot 22\ MoO_3 \cdot 2\ MoO_2 + H_2O$.—10 gm. of phospho-24-molybdic acid were dissolved in 50 cc. of H_2O. 5 cc. of 40 per cent HI and 5 gm. of $NaHSO_3$ were added. When all the sulfite had dissolved, 10 gm. of NH_4Cl were stirred in. The amorphous precipitate was filtered, washed with NH_4Cl until free from sulfate, and finally once with water. Blue powder. It lost 11.07 per cent of its weight on ignition. By calculation 1 gm. of the substance was equivalent to 0.8541 gm. of MoO_3. 0.3268 gm. of the substance suspended in water required 15.05 cc. of 0.02 N $KMnO_4$ for the titration. Whence 24 MoO_3 = 20.

3 $(NH_4)_2O \cdot P_2O_5 \cdot 20\ MoO_3 \cdot 4\ MoO_2 + H_2O$.—If in the above experiment the reacting mixture was allowed to stand 24 hours, the reduction would proceed further. The resulting product was quite soluble and was separated from the solution by saturation with NH_4Cl. A deposit of black precipitate was formed on standing. It was filtered, washed with 20 per cent NH_4Cl solution until free from sulfate, and finally once with water. The preparation lost 15.55 per cent of its weight on ignition. By calculation 1 gm. of the substance was equivalent to 0.8110 gm. of MoO_3. 0.2668 gm. required 24.85 cc. of 0.02 N $KMnO_4$ for titration. Whence 24 MoO_3 = 40.

Application of Complex Acids in Analytical Chemistry.

Phosphomolybdic and phosphotungstic acids have been used largely in organic analysis. The readiness with which the complex acids undergo reduction and the intensity of the color of the

resulting products should render them widely useful also in the analysis of inorganic compounds. The suggestions which follow are intended to illustrate the many uses to which the complex acids may be put. The mixed complex acids of the 1:18 series prepared as described above combine the stability of the pure tungsten compound and the sensitiveness of the pure molybdenum compound, and for use as color reagents they cannot be surpassed.

The complex acid solution should of course give no color when rendered alkaline. Sometimes the complex acid solution assumes a greenish tint due to some reduction by organic dust. This is of no practical consequence, but it can be removed by a few drops of bromine water. The excess of the bromine should of course be boiled off.

1. Detection of Copper.—Cuprous copper reduces the complex acid. The color of the reduction product is so intense that one part of Cu in 5,000,000 parts of water gives an unmistakable test. It is at least 50 times as sensitive as the ferrocyanide reaction. To apply the test to cupric copper proceed as follows:

To the copper solution add a few drops of 5 per cent potassium cyanide solution, acidify with HCl, followed by a few drops of the solution of the complex acid ("phenol reagent"). Mix. If more than a trace of copper is present the solution will become blue at once. But if only a trace of copper is present, the blue color will be obscured by the yellow color of the complex acid. Render the solution alkaline with saturated sodium carbonate solution, whereupon the unreduced yellow complex acid is transformed into a colorless salt. If copper is present the solution will now be blue.

The complex acids react also with ferrous salts, hydrogen sulfide, hydrogen iodide, sulfur dioxide, stannous chloride, etc., and its usefulness for the detection as well as for the colorimetric determination of these substances is apparent.

2. Detection of Phosphoric Acid.[8]—Hydrogen iodide reduces phosphomolybdic acids but not simple molybdic acid in the cold. Since the phospho-24-molybdic acid is formed from phosphoric acid and molybdic acid even in the cold, the presence of phosphoric acid can be tested with the aid of hydrogen iodide. The test may be made as follows:

[8] Arsenomolybdic acids probably react like phosphomolybdic acids.

To the solution to be tested, add 1 to 2 cc. of 2 per cent pure ammonium molybdate solution, then 2 to 3 cc. of 10 per cent potassium iodide solution, 1 cc. of 10 per cent $NaHSO_3$ solution, and finally 1 to 2 cc. of concentrated HCl. Mix the solution and allow to stand. The color develops slowly when the amount of phosphoric acid present is small, but the solution will assume a greenish tint after 10 or 20 minutes even when only a trace of phosphoric acid is present. The bisulfite destroys the iodine liberated and hastens the reaction, but it imparts to the solution a yellow color and tends to obscure the blue color of the reduced phospho-24-molybdic acid. Render the solution alkaline with saturated sodium carbonate solution. The yellow color will now disappear and a pure blue color will be obtained. One part of phosphorus in 1,000,000 parts of water gives unmistakable test. It is well to carry a blank to insure that the reagents used are free from phosphoric acid.

3. Colorimetric Determination of Phosphoric Acid.—The qualitative reaction for phosphoric acid can be made the basis of a colorimetric method for the determination of that substance. The writer expects to apply the method to biological fluids and to describe the details elsewhere. The provisional procedure may be given as follows:

To the unknown solution containing 0.1 to 0.2 mg. of P in a volume of about 5 cc. add 5 cc. of each of the following solutions: 10 per cent sodium bisulfite, 10 per cent potassium iodide, 2 per cent ammonium molybdate, and 1:1 HCl. Add the same solutions to 5 cc. of standard phosphate solution containing 0.15 mg. of P. Mix. Cover the flasks (graduated at 50 cc.) with watch-glasses and allow to stand for 2 hours. Then add to each flask 10 cc. of 20 per cent sodium carbonate solution, shake to hasten the escape of CO_2, dilute volume, mix, and compare the color values in a colorimeter (Duboscq). The color developed at the end of 2 hours is not the maximum, but it is sharply proportionate to the amount of phosphoric acid within the range from 0.1 to 0.22 mg. of P.

4. Indicator in Oxidation-Reduction Titrations.—An inside indicator for oxidation-reduction titrations is a long felt want in analytical chemistry. The advantages of the dichromate solution are offset by the inconvenience of the outside indicator. If to a ferrous salt solution a few drops of the mixed complex acid solution are added, the solution will be colored blue until all the ferrous iron is oxidized. Unfortunately the green color of the chromium salt tends to obscure the end-point, and satisfactory results can be obtained only when the amount of chromium involved is small (about 30 cc. of 0.02 N bichromate solution),

and then it is also necessary to have a blank by the side for comparison.

If no colored compound is produced in the oxidation-reduction reaction, the end-point is then very sharp. This is the case when a ferrous solution is titrated with a solution of hydrogen peroxide. The following experiment may be cited.

20 cc. of 0.05 N ferrous ammonium sulfate (in 10 per cent H_2SO_4) solution were measured into an Erlenmeyer flask, and three drops of the complex acid solution were added. An approximately N/15 solution of hydrogen peroxide was then run into the iron solution until all the blue had disappeared and a pure yellow color (due to complex acid) was obtained. 29.98 cc. of H_2O_2 were used. 30 cc. of the same solution required exactly 20 cc. of standard 0.05 N $KMnO_4$ solution for the titration. In this experiment a solution of ferrous salt previously titrated with a slight excess of H_2O_2 was placed by the side for comparison when the end-point was approached.

Hydrogen peroxide does not of course keep well enough to be depended upon for constant use, unless it is standardized with $KMnO_4$ whenever used. The writer offers the suggestion only to indicate the possibility of this novel use of the complex acid.

SUMMARY.

1. There are two series of complex phospho-acids of molybdenum and tungsten. In one series the ratio $P_2O_5 : MO_3$ is as 1:18; in the other, as 1:24. The "uric acid reagent" and "phenol reagent" of Folin and Denis contain acids of the former series.

2. Distinguishing reactions for the different complex acids are given.

3. Conditions of the formation of complex acids have been studied, and simple methods for the preparation of all the complex acids have been worked out.

4. Two phospho-18-tungstic acids, possibly isomeric forms, have been prepared.

5. A large number of complex acids containing both molybdenum and tungsten are shown to exist, and the method of their preparation is illustrated by an example.

6. Complex acids give on moderate reduction a number of new complex acids containing lower oxides of molybdenum and tungsten. The possibility of isomerism in these compounds is discussed.

7. New applications of the complex acids in analytical chemistry are illustrated by their use for the detection of minute quantities of copper and of phosphoric acid, for the colorimetric determination of phosphoric acid, and by their use as inside indicator for oxidation-reduction titrations.

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REACTION OF MILK IN RELATION TO THE PRESENCE OF BLOOD CELLS AND OF SPECIFIC BACTERIAL INFECTIONS OF THE UDDER.

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INTRODUCTION.

In a previous paper (Van Slyke and Baker, 1919) it has been shown that certain relations exist between the reaction of apparently normal milk and its chemical composition; and brief reference is made to the relation between the reaction of milk and the presence of leucocytes and streptococci. In conclusion it is stated that the conditions are such that they support the idea that the low hydrogen ion concentration occasionally found in freshly drawn milk is due to the presence of blood serum in the milk. Because of the importance of this suggestion in explaining bacteriological and physiological problems, the biological data that have a bearing on this theory are discussed in the present paper.

It is well known that under abnormal conditions not only blood serum, but blood itself may enter without change into milk as secreted. In some cases the blood enters because of hemorrhages due to injury or natural rupture of blood vessels; but in other cases the blood enters because of pathological conditions that are caused by specific bacterial infections. The commonest of the pathogenic organisms that cause the breaking down of the glandular tissue are the pathogenic streptococci, and this type of infection is the only one that has been studied during the present investigation. It should not be forgotten, however, that pathogenic staphylococci, organisms of the colon group, *Bacillus pyogenes*, the organism of bovine tuberculosis, and species of *Actinomyces* have all been described as causing similar infections (Wall,

1918). Recently attention has been called to infections of the udder with *Bacterium abortus* by Schroeder and Cotton (1911).

It has been abundantly established by a comparison of the composition of blood serum and of normal milk that the nutrient materials of the latter are quite different from those of the former. Normal milk solids are the product of the secretory activity of the glandular epithelium. The cells in this epithelium secure their raw materials from the blood serum, or more directly from the lymph, and transform them into the fats, casein, lactose, and other solids of the milk. Few substances other than water and some of the mineral salts pass unchanged into milk during normal secretion.

The situation in regard to the formed elements in the blood such as the red and white corpuscles differs from that just discussed. The red cells do not pass into the milk under normal conditions, while leucocytes have been shown to be so universally present that they must be regarded as a normal constituent of market milk (Breed, 1914). The numbers of these leucocytes are highly variable, and in milk of normal type they occasionally occur in such small numbers as to be practically absent. Even in the majority of samples of market milk, the number of these leucocytes is not large as compared with the number present in the blood; *i.e.* less than 500 per cm. for the majority of milk samples as compared with a normal of 7,500 per cm. for blood.

Accompanying these leucocytes in milk, there are smaller numbers of fat-laden epithelial cells (so called colostral corpuscles) and nuclei which represent the wastage from the lining epithelial found in the alveoli and ducts of the udder. They are, of course, not found in blood.

All gradations exist between evidently abnormal and strictly normal milk; and conditions that are significant in interpreting the phenomena of milk secretion are found in this intermediate region. Many samples that appear normal to the unaided senses show evidences of slight deviations from normal by chemical or microscopic analysis.

HISTORICAL.

Probably the first worker to show that milk from infected udders was abnormal in chemical composition and less acid in reaction than normal milk was Storch (1884, 1889). Similar results have been obtained by several later investigators; and Rullmann (1911) has shown that milk having a high leucocytic count likewise has a low titratable acidity. The later author sought the explanation of this lowered acidity in the presence of alkali-forming bacteria that more than counteracted the effect of the acid-forming streptococci, but could find nothing to confirm this view.

Fetzer (1912) also discusses the changes in milk caused by pathological conditions. He reaches conclusions essentially the same as those of Storch.

Höyberg (1911), working with his rosolic acid-alcohol test for determining the reaction of milk, concludes that excessive numbers of leucocytes in milk are accompanied by a lessened acidity which he believes (as does Storch) is caused by the passage of blood serum into milk without change. He finds that, whereas a mixture of equal parts of cow blood serum and normal milk gave a deep red reaction with the rosolic acid-alcohol reagent, a mixture containing five parts of normal milk to one of blood serum gave a normal reaction for milk. He regards this as indicating that relatively large amounts of unchanged blood serum must pass into milk where the reaction is decidedly less acid than normal.

Höyberg's test is made by adding 5.5 cc. of his rosolic acid-alcohol reagent to 5 cc. of milk in a test-tube. The color obtained is compared with the colors of a color chart. The reagent is prepared by adding 4.5 cc. of a 1 per cent alcoholic solution of rosolic acid to 5.0 cc. of 96 per cent alcohol. Though he gives no warning, care should be taken that the alcohol is acid-free.

Bahr (1913–14) has tried Höyberg's test and reports that he has secured fairly satisfactory results with it in detecting cows giving milk with high leucocyte and bacterial content. He, like Rullmann, sought the explanation of the decreased acidity in the presence of alkali-forming bacteria, but without success. He comes to the conclusion that Höyberg is mistaken in assuming that large quantities of unchanged blood serum are present in the less acid milk, showing that the addition of sufficient blood serum to cause the observed changes in reaction introduces an improbable amount of coagulable proteins. He seeks the explanation of the lessened acidity in the passage of tri-basic sodium phosphate from the blood into the milk without change into mono-basic phosphate.

Turning now to the papers dealing with the relation between the leucocytes and the streptococci, we find practically universal agreement that infections of the udder with pathogenic streptococci are accompanied by the presence of large numbers of leucocytes in the milk. This literature has already been reviewed (Breed, 1914) and need not be again discussed other than to point out that while Russell and Hoffmann (1907) found a practically constant relation between these two things, yet they noted

several instances where high cell counts occurred without detectable infection with streptococci. In the two cows the milk of which was studied during a lactation period by Hastings and Hoffmann (1909), there were consistent high counts of cells accompanied by a streptococcic infection. The latter, however, produced no evident change in the normal appearance of the milk. Likewise in the work on cell studies previously reported (Breed, 1914, p. 167), the history of the fluctuations in cell content of the milk of a cow that had recently freshened was followed for 24 days. In this case, though the milk contained many cells at some milkings, there was no detectable infection with streptococci. The milk was of normal appearance after the colostral period had passed.

Some recent work by Cooledge (1918) suggests that some of the cases where high cell counts have been obtained in milk of normal appearance are caused by infections with *Bacterium abortus*. These organisms are not so readily recognized in milk as are the streptococci, and are now known to occur quite frequently in fresh milk. Purely physiological disturbances in the secretory processes such as occur during the colostral period may also cause fluctuations in cell counts.

Technique Used.

In the present work the reaction of the milk has ordinarily been measured by the use of the brom-cresol purple test described by Baker and Van Slyke (1919). It was felt that this would be a more delicate indicator of changes of reaction of normal milk than the rosolic acid indicator used by Höyberg; but, in order to test the matter, a comparative trial was made of the two indicators with samples of fresh milk drawn from cows in the Station herd which were selected in such a way that a series of samples was obtained with varying reaction.

For comparison with the two indicators named, three other indicators were used, all of which are sensitive to changes where pH values are higher than those of strictly normal milk, but which are not so sensitive where the pH values are those of normal milk (6.50 to 6.60). These were saturated alcoholic solutions of rosolic acid, phenol red, and brom-thymol blue. In all cases except with Höyberg's reagent, one drop of the indicator was added to 3 cc. of milk. With the latter, the proportion was kept as indicated by the author, namely 5.5 cc. to 5 cc.

At the same time the reaction of the samples was determined electrometrically.

As a result of the color tests on the eleven samples (Table I), four observers were found to agree on the arrangement of the samples according to differences in shade of color. This sequence in color shades was found to agree with the sequence established electrometrically in all cases where the reaction fell within the known sensitive range of the indicator.

TABLE I.

Reaction of a Series of Milk Samples.

Results arranged in sequence according to pH values for comparison with the colors obtained with various indicators.

Sample No.	Reaction value.*	Sequence as established by the shade of color of indicator.					Notes.
		Brom-cresol purple †	Brom-thymol blue.‡	Phenol red §	Rosolic acid. ‖	Höyberg's reagent ‖	
	pH						
1	6.51	1	1	1	1	3	Nos. 1 to 7 normal in appearance and from apparently normal udders.
2	6.53	2	1	1	1	4	
3	6.54	3	1	1	1	5	
4	6.58	4	1	1	1	2	
5	6.64	5	2	1	1	7	
6	6.73	6	3	2	2	1	
7	6.78	7	4	3	3	6	
8	6.87	8	5	4	4	8	Sample normal; but from a quarter previously affected with garget.
9	6.90	9	6	5	5	9	From inflamed quarter. Normal in appearance.
10	6.95	10	7	6	6	10	Similar to No. 9.
11	7.08	11	8	7	7	11	From quarter badly affected with garget. Abnormal in appearance.

* Determined electrometrically.
† Color change from light blue to dark purple-blue.
‡ " " " greenish yellow to green.
§ " " " yellow-orange to red.
‖ " " " pink to dark red.

However, the curdling produced by the alcohol in Höyberg's reagent caused irregularities in color in the less sensitive range of rosolic acid (pH between 6.50 and 6.79). It was only samples showing greater pH values than this (Samples 8 to 11) that showed

a regular sequence in color change. All the latter samples were from quarters that were either inflamed at the time or had been affected with garget previously.

From these results it was concluded that whereas Höyberg's reagent was sufficiently sensitive to pick out milk that was decidedly less acid than normal, it was not a satisfactory test for the purposes in hand, nor as good for general use as the brom-cresol purple test.

Differential leucocyte and epithelial cell counts have been made by the method originally described by Prescott and Breed (1910). In making the counts, all the small cells bearing a close resemblance to mononuclear, polymorphonuclear, and polynuclear leucocytes were included in the one group, while all the large vacuolated cells of the colostral corpuscle type and all the large separate nuclei probably originally derived from epithelial cells were counted in a second group. This classification was followed as it is *a priori* improbable that any close relation exists between the reaction of milk and the amount of epithelial cell debris present.

The presence or absence of streptococci has been determined in each case by a microscopic examination of the stained dried milk as freshly drawn. If this examination was negative, a second microscopic examination was made after incubating the milk sample for 24 hours at 37°C. Streptococci have been reported as present whenever it has been possible to find cocci in chains which consisted of more than four individuals to the chain. Some chains were short, containing scarcely more than the minimum number of organisms; but the majority were long; *i.e.*, contained twenty to thirty or more individuals in the chain.

No conclusions should be drawn concerning the number of samples in any class as the samples were taken from animals known to be infected with streptococci, or giving milk with a reaction less acid than normal in one or more quarters.

The samples were drawn aseptically from individual quarters of the udder and were examined for reaction at once. They were taken after the milking was approximately half completed. The animals in the Station herd are Jerseys, with some grade Jerseys, while the animals in the other herds were Holsteins mixed with common stock.

Data and Observations.

Observations were made of the reaction and cell and streptococcic content of 63 samples of milk from cows in the Station herd; and of 61 similar samples from animals in two herds in the neighborhood.

As it is difficult to pick out the relations existing between the reaction, cell content, and streptococci from the results as obtained, they have been arranged in Tables II, III, and IV in such a way as to contrast the reaction with the leucocyte content, the epithelium cell content, and the presence of streptococci. Finally, the averages of these tables have been combined in Table V for convenience in comparison.

Even a casual examination of the detailed results shows that a relation exists between the number of leucocytes present and the reaction of the milk. This appears in a more striking way in Table II in which the results are arranged according to a scale of decreasing acidity. From this it appears that the highest leucocyte count noted from milk with the reaction of Group 1 (pH values between 6.50 and 6.60) was 330,000 per cc., while the average count for the thirty-seven samples in this class was only 46,000. Fourteen of these samples contained so few leucocytes that none was observed in the microscopic examination of $\frac{1}{3,000}$ cc.

In the twenty-one samples in which there was a slight but perceptible darkening of the brom-cresol purple (pH values between 6.60 and 6.68), it will be noted that whereas four samples contained so few leucocytes that none was seen in the microscopic examination, two samples contained in excess of 1,000,000 per cc. (maximum 1,440,000). The average leucocyte content of the twenty-one samples was 215,000 per cc.

With certain exceptions to be noted presently, there is a gradual, though somewhat irregular, increase in the number of leucocytes with decreasing acidity as shown by the regular increase in the average leucocyte count for each group of samples. This reaches its climax in the ten samples of milk in which the pH values are greater than 7.00. In these the lowest leucocyte count was 1,980,000, and the highest, 40,900,000 per cc. The average count for this group was 16,800,000 per cc.

Two counts were so widely divergent from the usual conditions that they were not included in the averages. The two unusual samples fell in Group 5 (pH values 6.84 to 6.92). One gave a leucocyte count of 20,000,000 and the other 33,800,000 per cc.

TABLE II.

Comparison between the Reaction and the Leucocyte Content of Milk of Normal Appearance.

Leucocyte counts given in thousands per cc.

Group 1. pH 6.50–6.60.		Group 2. pH 6.60–6.68.	Group 3. pH 6.68–6.76.	Group 4. pH 6.76–6.84.	Group 5. pH 6.84–6.92.	Group 6. pH 6.92–7.00.	Group 7 of Table III. pH 7.00+.
thousands	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*
90	0	1,440	220	1,620	2,460	3,900	21,600
60	10	0	380	4,110	5,100	1,600	34,000
30	70	30	190	240	320	4,000	40,900
0	110	410	70	3,690	20,000	10,000	1,980
0	90	20	2,550	90	1,520	3,000	4,300
0	0	0	1,510	300	380	3,180	Innu-
120	0	0	0	270	8,100	1,740	merable.
0	180	250	360	360	33,800	7,380	4,000
30	0	60	120	2,580	540	8,400	7,800
0	30	40	1,020	120	16,500	3,000	15,300
30	30	110	2,040	300	1,770	5,000	21,000
0	330	160		1,120			
30	0	1,030		900			
0	0	220		510			
120	0	0		800			
110	120	60		810			
10		60		1,000			
40		60					
40		20					
10		180					
30		360					
Average	46	215	770	1,100	8,230 4,080*	4,650	16,800

* Two excessive counts omitted from the average.

These counts are both greatly in excess of the average count for samples in this group. In the first of these cases, a sample taken from the same quarter a few days previously had given an almost identical count (21,600,000 per cc.) with a less acid reaction (pH 7.00 +).

In spite of these and other less noticeable exceptions, the observations are in general accord with the theory that decreased acidity is correlated with increase in the number of leucocytes present in fresh milk. Minor irregularities are explainable by inaccuracies of technique, while some of the larger ones are undoubtedly due to the influence on the reaction of the production of acid from lactose by streptococci. No satisfactory explanation of these relations is evident if the leucocytic nature of the cells is denied as is done by Winkler (1908) and by Hewlett, Villar, and Revis (1909–1913).

The relation between the amount of cellular debris of epithelial origin and the reaction as shown in Table III does not appear to be as striking as that with the leucocytes. Where the results are arranged according to reaction and the cell counts averaged, it is evident that there is more epithelial cell debris in the samples with the lowest acidity. Yet the increase in amount of cell debris with decreasing acidity is not regular. This suggests that the relation between the two things is not a close one, and agrees with the conception that inflamed conditions are likely to cause a discharge of an increased but irregular amount of epithelial cell debris.

In considering the possible relation between the reaction and the presence or absence of streptococci, the conditions under which such a condition might exist should be kept in mind. The pathogenic streptococci are well known inciters of inflammatory processes in animal tissues, and are common causes of such troubles in udders. During any disturbance of this type in the udder, the leucocytes tend to congregate in large numbers. Both the leucocytes and an accompanying serous exudate probably pass through the glandular epithelium under these conditions. At first there would be little, if any, actual disorganization of the glandular epithelium but eventually, as shown in the excellent figures drawn by Ernst (1913), the lining of certain alveoli in limited areas would actually break down, thus allowing the direct entrance of lymph, or even blood serum, by the rupture of the walls of the vessels carrying these body fluids. In the later stages, this disorganization would involve large areas, even entire quarters of the udder. The secretion would no longer have the appearance of normal milk under such conditions.

During the period previous to the actual rupture of the epithelium and vessels, it seems probable that the normal life processes controlling the entrance and elaboration of the blood nutrients in the gland cells would be weakened or modified. A natural change would be for the readily dialyzable substances of the

TABLE III.

Comparison between the Reaction and the Epithelial Cell Content of Milk of Normal Appearance.

Epithelial cell counts given in thousands per cc.

Group 1. pH 6.50–6.60.		Group 2. pH 6.60–6.68.	Group 3. pH 6.68–6.76.	Group 4. pH 6.76–6.84.	Group 5. pH 6.84–6.92.	Group 6. pH 6.92–7.00.	Group 7. pH 7.00 +.
thousands	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*	*thousands*
30	0	240	590	210	150	1,200	1,230
0	60	0	640	270	360	420	1,260
60	160	0	370	90	200	660	0
0	490	970	110	420	570	210	180
0	330	40	220	60	660	450	270
0	0	0	780	120	570		330
0	0	60	0	30	1,870		300
30	30	270	90	480	3,300		930
30	90	150	0	300	840		
60	0	120	570	12	390		
60	60	220	240	30			
30	0	420		120			
0	0	660		90			
10	60	490		60			
0	0	60		150			
580	0	60		180			
180		90					
190		90					
140		0					
70		30					
50		30					
Average	75	190	330	160	890	590	560

blood, such as the mineral salts, urea, glucose, and amino-acids to pass into the milk with the serous exudate in increased amounts without undergoing the usual processes of elaboration. However, the chemical findings previously reported (Van Slyke and Baker, 1919) indicate that even under these conditions the glucose is

transformed into lactose (see also Porcher, 1905). If, however, the mineral salts of the blood (especially sodium bicarbonate and basic phosphates) pass into the milk, they would reduce the hydrogen ion concentration, even though the remaining substances of the blood serum did not enter.

The entrance of some other subtances in unaltered condition early in this process is indicated by the fact that fibrin is readily demonstrated by appropriate stains in the less acid milk. This would indicate that, under the intermediate conditions before there is actual entrance of unaltered serum, there is a considerable period during which there is an entrance of modified or partially elaborated serum.

Another factor having a direct influence on the hydrogen ion concentration which ought not to be overlooked is the formation of acid by the streptococci by the fermentation of glucose or lactose. From the fact that the reaction of the streptococcus-infected milk is less acid than normal, it is evident that there must be a surplus of the substances causing a reduction in hydrogen ion concentration. As long as the new materials are being secreted the acidity is ordinarily neutralized; but as soon as the milk is drawn the streptococcic fermentation quickly changes the reaction to an acid one. This was evident in several samples where flocculent masses of bacteria settled to the bottom of sample tubes to which brom-cresol purple had been added. Within 30 minutes acid areas showed about each of the flocculent masses.

Even a casual study of the detailed data shows that streptococci were usually present in those cases where there were excessive numbers of cells accompanied by decreased acidity. The correlation between decreased acidity and the presence of streptococci is shown clearly in Table IV. In this summary it will be seen that whereas only one sample out of thirty-six (3 per cent) with a normal reaction (pH values from 6.50 to 6.60) was found to contain streptococci, the percentage of streptococcic infection increased regularly with decreasing acidity. This reached its climax in Group 7 (pH values 7.00 +) all of which contained streptococci.

Certain exceptional conditions are worth a further word of explanation. The single sample of milk with normal reaction that contained streptococci contained so few of them that they did

not appear in a microscopic examination of the milk until after incubation for 24 hours at 37°C. The cell count was likewise zero for $\frac{1}{3,000}$ cc. Evidently the infection in this case was dormant or so localized that it did not affect the reaction.

TABLE IV.

Comparison between the Reaction and the Presence or Absence of Streptococci in Milk of Normal Appearance.

Group 1. pH 6.50–6.60.		Group 2. pH 6.60–6.68.	Group 3. pH 6.68–6.76.	Group 4. pH 6.76–6.84.	Group 5. pH 6.84–6.92.	Group 6. pH 6.92–7.00.	Group 7. pH 7.00 +.
−*	+	−	+	+	+	+	+
−	−	−	+	+	+	+	+
−	−	−	+	−	+	+	+
−	−	−	−	+	−	+	+
−	−	−	+	+	−	+	+
−	−	−	+	+	−	+	+
−	−	+	−	+	+	+	+
−	−	+	−	+	+	+	+
−	−	+	−	+	− (?)	−	+
−	−	−	−	−	+	+	+
−	−	−	−	−		+	
−	−	−	+	−			
−	−	−	+	−			
−	−	−					
−	−	−					
−	−	−					
−	−	+					
−		−					
−		−					
−		−					
Per cent present	3	19	54	62	60	91	100

* Streptococci present +, streptococci absent −.

The single sample in Group 6 (pH values 6.92 to 7.00) that showed no streptococcic infection was taken from an udder where all the other quarters were infected with streptococci, one of them giving abnormal gargety milk. The leucocyte count was high (1,740,000 per cc.) and the conditions indicate that the inflammation in the other quarters had influenced the secretion in the uninfected quarter.

In one of the samples of Group 5 (pH values 6.84 to 6.92) reported as negative for streptococci a question mark has been added because a sample taken from the same quarter a few days later contained streptococci. The other negatives in this group may likewise have been caused by failure to find the streptococci, or they may have been caused by infection with other pathogenic organisms. Some were from cows where streptococcic infections were present in other quarters, while some were from cows where no streptococcic infection could be detected with the technique used.

TABLE V.

Comparison between the Combined Averages of Tables II, III, and IV.

Group	1	2	3	4	5	6	7
pH values	6.50–6.60.	6.60–6.68.	6.68–6.76.	6.76–6.84.	6.84–6.92.	6.92–7.00.	7.00 +.
Average leucocyte count, *thousands per cc*	46	215	770	1,100	4,080	4,650	16,800
Average epithelial cell count, *thousands per cc.*	75	190	330	160	890	590	560
Samples containing streptococci, *per cent*	3	19	54	62	60	91	100

When the combined averages are compared as in Table V, it is seen that the conditions governing the reaction, presence of leucocytes, epithelial debris, and streptococci are all in harmony with the idea that decreased acidity is primarily caused by the entrance of the alkaline substances contained in the lymph and blood serum. It is not probable that entirely unaltered blood serum enters until the inflammatory processes have gone so far that there is actual rupture of the blood vessels. Accompanying the entrance of these alkaline substances there is a fairly regular increase in the entrance of leucocytes, and a less regular increase in the amount of epithelial debris. In a majority of the samples examined, the primary cause of these changes appeared to be an infection with streptococci.

CONCLUSIONS.

In the previous paper by Van Slyke and Baker (1919) it is suggested that decreased acidity of fresh milk is due to the direct filtration of blood serum into the milk as secreted. In support of this view it is pointed out: (1) that this view is in harmony with the changes in composition that accompany decreased acidity; (2) that it is in agreement with the hydrogen ion concentration of normal milk (pH values 6.50 to 6.60) and that shown by blood serum (pH about 7.60); and (3) that it harmonizes with an increased CO_2 content of the less acid milk, normal milk containing about 10 per cent by volume of CO_2 and blood serum, 65 per cent.

Further proof of the presence of blood serum was furnished by the presence of fibrin as shown by fibrin stains. This normal constituent of blood does not appear in normal milk.

The only observed chemical relation that was out of harmony with the view that unchanged blood serum caused the low acidity of certain samples of milk was the fact that no glucose could be demonstrated in several samples in which it should have been present in appreciable quantities if this were the case. At that time it was suggested that further investigations were desirable.

From the new data here given, and from histological and physiological considerations, it appears that a more exact statement of the case would be that the infection causes the entrance of a serous exudate derived by the gland cells directly from the lymph rather than from blood. This serous exudate is neither exactly like blood serum nor milk. When the infection has proceeded to the place that actual rupture of the vessels occurs then unchanged lymph and blood would enter. The modifications in the secretions may be due to a weakening of the secretory activity of the cells or the exudate may have a definite protective function against the bacterial infection, or both conditions may hold simultaneously.

The processes involved are so complicated that it is little wonder that the matter is not clear even with the data at hand. It seems quite probable, however, that the increased CO_2 content of the milk samples having a lowered acidity is correlated with the entrance of bicarbonate from the blood. The acidity of the milk

would also be lessened by the entrance of increased amounts of basic phosphates or even of albumins. Changes in the secretion of the milk in relation to these substances could easily explain the changes in reaction actually noted without assuming the entrance of excessive amounts of unchanged blood serum or lymph. While these chemical changes are still only partially understood, the data establish the fact that the modifications of the secretion are correlated with an increased entrance of the leucocytes into the lumina of the alveoli. Accompanying this change in the secretory processes there also seems to be an increasing amount of wastage from the glandular epithelium.

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RELATION OF UROCHROME TO THE PROTEIN OF THE DIET.

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The literature dealing with the specific yellow coloring matter of the urine, the urochrome of Thudichum and of Garrod, shows, in addition to a number of more or less effective methods of preparation, an almost equal number of theories as to the probable origin and significance of that body. Since 1798, when Fourcroy and Vauquelin (1) first attempted to connect the yellow color of the urine with the excretion of urea, various workers have speculated with different results as to its origin. For an excellent historical review of the subject up to 1907 the reader is referred to St. Dombrowski's paper (2). For the purposes of the present discussion it is necessary only to show the differences of opinion that exist with regard to the origin of urochrome by citing briefly the conclusions of some of the more recent observers. Thudichum (3), although at first in favor of Maly's (4) theory of the urobilin origin of urochrome, later expressed his doubts as to its accuracy; Garrod (5), on the basis of his active aldehyde reaction, thought urobilin to be its precursor; St. Dombrowski (2) held the body proteins responsible for its appearance, and Weisz (6), as a result of very extensive investigations, concluded that urochrome is an "Abbauprodukt des Zellstoffwechsels" and thought that he had in its excretion a measure of the cell metabolism of the body; Klemperer (7), in 1903, made the following statement: ". . . . at present I am inclined to believe that urochrome is a direct derivative of the blood pigments and is synthesized by the kidney," adding further that "an influence of the diet upon the excretion of urochrome has never been observed." The last statement is important here not only because it is the only positive statement which can be found in the literature with regard to the effects of the diet, but also because the present investigation shows it to be erroneous. Palmer and Cooledge (8), in a thorough examination of Garrod's results, state that urochrome probably originates from the blood rather than from the bile. No conclusions can be drawn from this work (referring to their own work on the identity of lactochrome with urochrome) as to whether urochrome is a decomposition product of protein or of blood pigment.

The present investigation was undertaken at the suggestion of Professor W. R. Bloor, with the intent of correlating the facts

brought out by previous observers and, perhaps, to establish more firmly the origin of the substance in question.

The thought that the protein intake may in some measure be responsible for the color of the urine seemed a promising one and was followed up with a series of experiments in variations of protein intake. The method of procedure was as follows. A 24 hour sample of urine was collected and a small portion of it filtered and compared in the Duboscq colorimeter with an arbitrary standard of 3.2 mg. of Bismarck brown and 8 mg. of Echtgelb Y[1] in 1 liter of water. The color corresponded closely to that of the normal urine, and a value of one unit per cc. was assigned to it. Another determination was made by adding 5 cc. of a 20 per cent solution of lead acetate to 25 cc. of the urine and comparing the filtrate with another arbitrary standard of 3 mg. of Echtgelb Y in 1 liter of water. In this manner two determinations were made of each 24 hour sample, one on the total color of the filtered urine, and another on the urine from which the proteins and such colors as urobilin and uroerythrin had been largely removed by precipitation with lead acetate, and which contained, therefore, urochrome as the principal coloring matter. In calculating the color units of the precipitated urine, due allowance was made for the dilution with lead acetate.

The results of the experiments are contained in Tables I to VI.

From these tables it is evident that protein may influence, to a remarkable extent, the daily urochrome output. On a low protein diet the output of urochrome was reduced to less than one-half its normal amount. It is probable that the amount of urochrome which still remained in the urine after several days of low protein diet is due, in part at least, to the protein products of the cell metabolism. A diet of carrots, following a low protein diet, raised the urochrome output slightly (Table IV), due, perhaps, to the consequent slight increase in protein intake, but did not raise it as high as should be expected if such colors as carotin or xanthophyll were concerned with urochrome excretion. Table III shows that the amount of daily urochrome is perceptibly raised by a high protein diet; it is, however, also apparent that this increase is not proportional to the decrease which

[1] The dyes were obtained through the kindness of H. A. Metz and Company, San Francisco.

may be obtained on a low protein diet. This failure to increase the excretion of urochrome to a more marked extent may be caused by the inability of the body to deal with more than a certain limited amount of that substance. A gelatin diet, preceded and followed by a low protein diet, showed no increase (Table V) in urochrome excretion, probably because of the absence of the color-producing groups (aromatic series) in this

TABLE I.

Normal Protein Diet (10 Gm. of N).

24 hr. volume.	Color units.	
	Filtered urine.	Lead acetate urine.
cc.		
1,100	1,060	1,146
1,150	1,287	1,202
1,300	1,277	1,115
1,020	1,032	1,035
925	1,138	1,008

TABLE II.

Low Protein Diet (6 Gm. of N).

24 hr. volume.	Color units.	
	Filtered urine.	Lead acetate urine.
cc		
1,120	1,077	632
1,000	961	678
1,290	993	666
750	902	563
810	838	540

incomplete protein. In order to determine whether the urochrome excretion was constant from hour to hour, an examination of 2 hour samples of a high protein urine was made. Table VI shows that the output is variable and may in one 2 hour period be three times greater than in the preceding period.

The next logical step in this examination is, of course, the determination of the particular group or groups within the protein molecule to which this color is due. Lack of time has, unfortunately, so far prevented such an investigation. Some pre-

TABLE III.

High Protein Diet (14 Gm. of N).

24 hr. volume.	Color units.	
	Filtered urine.	Lead acetate urine.
cc.		
1,305	1,274	1,291
1,440	1,377	1,312
1,255	1,265	1,308
1,350	1,312	1,418
1,190	1,263	1,380

TABLE IV.

Carrot Diet Following Low Protein Diet.

24 hr. volume.	Color units.	
	Filtered urine.	Lead acetate urine.
cc.		
1,050	1,078	737
980	1,040	720

TABLE V.

Gelatin Diet Preceded and Followed by Low Protein Diet.

24 hr. volume.	Color units.		Total nitrogen.
	Filtered urine.	Lead acetate urine.	
Low protein diet.			
cc.			*gm.*
1,125	1,071	581	5.84
625	1,136	511	4.83
Gelatin diet.			
925	1,045	508	8.11
Low protein diet.			
840	996	523	6.02

liminary experiments in this direction led, however, to the discovery of a body in peptone (also in casein, but not in gelatin) which in every way corresponds to Garrod's urochrome and, incidentally, to Palmer and Cooledge's lactochrome. The method of preparation is essentially the same as that used by these workers in the preparation of urochrome and lactochrome.

TABLE VI.
High Protein Diet in 2 Hr. Samples.

Time.	24 hr. volume.	Color units.	
		Filtered urine.	Lead acetate urine.
	cc.		
8.10 a.m.	259	145	192
10.12 "	150	125	125
12.2 p.m.	175	110	113
2.4 "	100	160	188
4.6 "	70	85	61
6.8 "	110	104	195
8.8 a.m.	450	545	391
24 hrs.	1,305	1,274	1,291

A solution of 50 gm. of peptone in 100 cc. of water (with casein a preliminary digestion with HCl was necessary) was saturated with solid ammonium sulfate and filtered. The color was extracted from the filtrate by 95 per cent alcohol, which rose to the top as a highly colored layer and was syphoned off. The alcohol extract was poured into an equal volume of water and again extracted by saturation with ammonium sulfate. It was then evaporated on the steam bath after having first been cooled on a mixture of salt and ice to crystallize out most of the ammonium sulfate which had dissolved in the alcohol. After the product so obtained in the desiccator was dried, it was rubbed up in a mortar and placed in absolute alcohol over night. The absolute alcohol extract was removed next morning and the substance again treated with absolute alcohol. The two extracts were combined and the coloring matter was precipitated out of solution by addition of several volumes of ether.

The body so produced gave all the tests for urochrome (except Garrod's aldehyde test, which could not be applied because lack of time prevented the production of "active" aldehyde), which Palmer and Cooledge used to prove the identity between that substance and lactochrome. It is prepared by essentially the

same method; the solubility in water, alcohol, ether, chloroform, acetone, and benzene is the same; it is precipitated out of its water solution with silver nitrate, mercuric nitrate, and phosphotungstic and phosphomolybdic acids, and is not precipitated by ammonium sulfate or lead acetate. With a pine-splinter moistened with HCl it gives a striking pyrrol reaction, and its bromine derivative shows essentially the same characteristics as Salomonsen's (9) bromourochrome and Palmer and Cooledge's bromolactochrome.

If, in addition to the fact that the chemical reactions of this body—which we may call "protochrome," at least until its nature is more fully established—correspond in every way to urochrome, it is considered that a low protein (low protochrome) diet reduces the output of urochrome more than 50 per cent, the conclusion that the two bodies are identical seems reasonable.

SUMMARY.

1. A low protein diet has been found to reduce the daily urochrome excretion markedly. A high protein diet increases the excretion perceptibly. Gelatin or carrots when taken in the diet have no effect on the output.

2. A new body, protochrome, the chemical reactions of which are identical with those of urochrome, can be produced from some of the proteins.

3. All evidence points to the fact that urochrome, lactochrome, and protochrome are identical and, at least to a large extent, are derived from the food proteins.

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A CONJUGATED NUCLEIC ACID OF PANCREAS.

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A recent preliminary communication by Feulgen[1] on a conjugated nucleic acid of pancreas prompts me to publish my own results on the same subject.

Feulgen prepared a nucleic acid from pancreas by enzymatic digestion of β-nucleoprotein. This acid could be split into phosphoric acid, levulinic acid, furfurol, thymine, cytosine, guanine, and adenine. He found that an alkali salt of guanylic acid could be precipitated with sodium acetate after digestion in alkaline solution. The nucleic acid was dextrorotatory. From these data Feulgen assumes his nucleic acid to be of a molecular composition between guanylic acid and tetranucleotide, and calls it "Guanyl-nucleinsäure."

As early as January, 1919, I isolated and described a "Guanyl-nucleinsäure" (Feulgen). In January of the same year the results were presented in a manuscript to the authorities entrusted with the appointment of a chemist at the Caroline Institute. I did not wish to publish my results, because of the incompleteness of the investigation at the time.

My results are essentially in accord with those of Feulgen, and the confirmation seems to be of sufficient value to justify publication.

Following is a summary of the most important of my results, up to January, 1919.

The method of preparing the nucleic acid had been worked out in the form described below.

[1] Feulgen, R., *Z. physiol. Chem.*, 1919, cvii, 147.

The calcium and sodium salts and the corresponding free acid had been analyzed. I had determined the ratio $\frac{N}{P}$ for the calcium salt as 1.9. After hydrolysis guanine, adenine, thymine, cytosine, and pentose were found. The ratio $\frac{\text{guanine}}{\text{adenine}}$ was determined to be about 3.

After an alkaline solution of the sodium salt of the nucleic acid had been warmed, cooled, and neutralized with acetic acid, a nucleic acid was precipitated, which could be identified as guanylic acid. A nucleic acid remained in the solution, which I could not at that time characterize. By determination of the alterations of the hydrogen ion concentration, the conductivity, and the freezing point after warming with a solution of caustic potassium hydrate, it was shown that acid groups had been freed; *i.e.*, that hydrolysis had taken place.

In the β-protein of pancreas (prepared according to Hammarsten) the ratio $\frac{\text{guanine}}{\text{adenine}}$ was stated to be about 3.

In the following pages the data, above summarized (collected during the year 1918), are reported together with the results of subsequent experiments.

The starting point of the present work was an attempt to isolate and fractionate the natural compounds of proteins and nucleic acids (nucleoproteins, nucleins) from pancreas, and then to produce nucleic acids from the different fractions. Different investigators have been looking for a third single nucleic acid of the type of the guanylic and inosinic acids, and I hoped to make progress in this field.

To this purpose dry pancreas was extracted with cold, dilute hydrochloric acid (0.06 N). From the undissolved matter the supposed nucleins were dissolved with alkali at a temperature of 0°C. and then precipitated with hydrochloric acid. The precipitate was purified by means of alternate dissolving and precipitating, and then dried with alcohol and ether.

The preparation dissolved perfectly clear on addition of alkali while the reaction was still slightly acid. For the sake of brevity I am going to call a neutral solution of the preparation in alkali "nuclein solution."

Lengthy attempts to get a constant fraction by means of salting out the nuclein solution with different neutral salts did not give satisfactory results. The highest fractions with ammonium sulfate showed, however, something of interest. From 80 per cent saturation with this salt up to complete saturation I got a precipitate which was easily soluble in water and which was precipitated by acids. It did not give any biuret reaction, and had (dried in the air) a high percentage of phosphorus (7.3 per cent). After hydrolysis with sulfuric acid a strong reaction for purine bases was noted. A splitting of nucleic acid-protein links had accordingly occurred, from the action of either the hydrochloric acid or the ammonium sulfate. This nucleic acid was not further investigated. The yield was very small, and in the meanwhile I had found that a nucleic acid could be isolated in ample quantities directly from a neutral nuclein solution by precipitation with calcium chloride. This calcium salt of nucleic acid, which was slightly soluble in water, was purified in different ways (see below), and from it the free acid and a calcium-sodium salt were prepared. All three preparations showed a ratio $\frac{N}{P} = 1.88$. For an ideal tetranucleotide the ratio $\frac{N}{P}$ is $= 1.69$, and for the guanylic acid, $= 2.26$. A combination of 2 molecules of guanylic acid with 1 molecule of tetranucleotide would give the ratio $\frac{N}{P} = 1.88$.

By warming the solution of the sodium salt of the nucleic acid with 1 per cent sodium hydroxide, the hydrogen ion concentration and the electrical resistance were raised, while the freezing point remained unchanged—positive signs of an hydrolysis with the forming of acid groups.

After neutralization of the hydrolyzed solution, a substance was precipitated, which could be identified as guanylic acid.

From these results it appears probable that the substance analyzed by me is composed of guanylic acid and tetranucleotide, united in genuine chemical combination. Possibly 2 molecules of guanylic acid and 1 molecule of tetranucleotide are here bound together.

A more complete description of the experiments follows.

EXPERIMENTAL.

Pieces of pancreas from cattle were washed *in situ* immediately after slaughter, via arteria pancreatica with isotonic salt solution in order to get rid of the main part of the blood. The pieces were then put into a vessel which was surrounded by a freezing mixture. On arrival at the laboratory they were freed from fat and lymph tissue, ground to a pulp, and put into 96 per cent alcohol. The alcohol was pressed out after 1 hour and the mass again mixed with 96 per cent alcohol. In this way the mass was extracted with 96 per cent alcohol three more times (after 3, 12, and 24 hours). Then it was treated twice with ether, dried in the air, and put into a ball-mill. The treatment with alcohol and ether is necessary for subsequent attainment of a filterable extract on treatment with sodium hydroxide.

600 gm. of the dusty, gray-white powder were extracted, with stirring, with 4 liters of 0.06 N hydrochloric acid for 24 hours at 6°C. The insoluble matter was then pressed to a solid cake, mixed with 1 liter of cold water, and again sharply pressed from the liquid. The solid pulp was ground with 2 liters of water at 0°C. and then, during cooling of the extraction vessel and stirring, mixed with a 0.06 N solution of sodium hydroxide at 0°C. in small portions. In these operations care was taken that the temperature did not exceed 0.5°, and that the mixture in the extraction vessel after each addition of sodium hydroxide did not give more than a weak bluish violet color with neutral litmus paper (about the same reaction which is obtained from a mixture of three parts of 0.1 N primary and seven parts of secondary phosphate). In the course of 4 hours 4,400 cc. of a 0.06 N solution of sodium hydroxide were added. The liquid, which then showed a neutral reaction, was strained through a closely woven linen cloth. The filtrate was strongly turbid and of a brownish yellow color. It was at once thrown onto a double layer of folded filter paper in a room cooled to 0°C. The filters were changed every day and new ones put on. In this way the whole extract could be filtered in 72 hours. The filtrate was straw-colored and nearly clear, but showed when standing during the filtration a slight turbidity. This was removed by a second filtration, which required but a few minutes. The filtrate was now entirely clear and had no foul or musty odor. To each liter were added 10 cc. of 10 per cent hydrochloric acid. Thereupon an abundant, coarsely flocculent, white precipitate appeared, which was centrifuged and washed several times in the centrifuge with 1 per cent acetic acid. The precipitate was then stirred with 1 liter of water at 0°, and mixed with a dilute solution of sodium hydroxide to neutral reaction. The liquid, which was somewhat turbid, was filtered once more at 0°C., the nearly clear filtrate precipitated with 10 cc. of 10 per cent hydrochloric acid, the precipitate washed repeatedly in the centrifuge with 1 per cent acetic acid, and then six times with 96 per cent alcohol. Finally it was washed twice with absolute alcohol, and then several times treated with ether, and each time was rapidly filtered on a suction filter. The preparation had, dried in the air, a nearly

white color. It was apparently neither hygroscopic nor soluble in water, but easily soluble in alkali, even at acid reaction. A 2 per cent solution was quite clear, had a yellowish color, and was precipitated as coarse flakes by acetic acid, hydrochloric acid, and calcium chloride.

A neutral solution of the preparation (for the sake of brevity in the following called "nuclein") showed at room temperature no tryptic activity. A nuclein solution, which had stood 24 hours at 20°, showed no alteration in the formol titratable nitrogen.

Destruction of the trypsin is effected by the action of 0.06 N hydrochloric acid on dry pancreas, as is shown by Table I.

After 24 hours (Nos. 1 to 6 at 6°C., No. 7 at 37°) Nos. 2 to 6 were neutralized with sodium hydroxide. To Nos. 1 and 7 the same amounts of NaCl were added that were generated in the other experiments. All the experiments were carefully brought to the same reaction to litmus. Nos. 1 to 4 and 7 were boiled for 5 minutes, cooled, and water was added to bring the solution up to 200 cc. Having stood for 24 hours at 6°, these samples were filtered and the nitrogen was determined. Nos. 5 and 6 were neutralized after the first 24 hours, 10 gm. of fibrin were added, and the mixture was once more digested for 24 hours at 37° and 6°C., respectively, then boiled for 5 minutes, brought to 200 cc. with water, filtered, and the nitrogen was determined in the filtrates. The analytical results are, of course, to be regarded as approximate.

Dry pancreas, fibrin, and hydrochloric acid gave 19 mg. of nitrogen. The result, No. 2 + No. 3 = 18.3, corresponds well to this. No tryptic digestion could have taken place here. The nitrogen is derived directly from water-soluble nitrogenous compounds. In No. 1, 52 mg. of N were found; *i.e.*, about 30 mg. were brought into solution through the influence of trypsin. Nos. 5 and 6 showed clearly that the influence of trypsin was neutralized through the treatment with hydrochloric acid. Only 2 to 4 mg. could be attributed to digestion, amounts which surely lay within the limits of error. The digestion of No. 7 yielded about 320 mg. This shows clearly—especially compared with the fact that no difference between the nitrogen values in Nos. 5 and 6 was noted—that the 24 hours influence of the 0.06 N hydrochloric acid at 6° had completely destroyed the trypsin.

In the filtration of the alkaline extract of pancreas, formol titrations were made in the filtrate every 12 hours. The amino nitrogen thus titrated did not increase. This shows that no breaking of peptide linkings had taken place. With the precautions which were taken in the preparation of the dry pancreas, I consider the risk of autodigestion of the pancreas brought to a minimum. Through the treatment with hydrochloric acid the trypsin was destroyed and the nuclein, which was free from active trypsin, could not be a product of enzymatic digestion.

In the nuclein (dried in the air), nitrogen and phosphorus were determined. Found: 17.09 per cent N; 5.61 per cent P.

Part I of the same preparation was dissolved in a solution of sodium hydroxide and Part II in ammonia. The solutions were precipitated with

a small surplus of hydrochloric acid, the precipitates washed with water, and dried with alcohol and ether. Found: (Part I) 19.09 per cent N; 4.69 per cent P; (Part II) 17.07 per cent N; 5.15 per cent P.

Also in preparations from different experiments (with the same method) the phosphorus varied between 4 and 5 per cent; the nitrogen, however, was constant. Considering that the phosphorus showed a great variation in nucleoproteins and nucleic acids, and the nitrogen a rather small one, it was probable that the preparation was composed of a mixture of protein-rich and protein-poor nucleins with perhaps nucleic acids.

A neutral 2 per cent nuclein solution in sodium hydroxide gave a strong biuret reaction with a bluish violet color, and was incompletely precipitated by acetic acid, hydrochloric acid, and alcoholic hydrochloric acid. Ammonium sulfate precipitated at a saturation of 25 per cent. The precipitation increased up to complete saturation. Calcium chloride and barium chloride gave strong precipitates.

The precipitation with $CaCl_2$ was more closely investigated. It appeared that maximum precipitate occurred in a 2 per cent nuclein solution on the addition of 0.5 per cent $CaCl_2$. At this point 23 per cent of the total amount of nitrogen was precipitated. Adding more $CaCl_2$ (up to 5 per cent $CaCl_2$ in the solution) gave no more precipitation.

Precipitation was equally complete, whether the nuclein solution was weakly acid or weakly alkaline. The calcium precipitate (in the following called Precipitate A) was washed several times with water, then repeatedly ground with alcohol, and treated on a suction filter, until no reaction for Ca or Cl was given by 50 cc. of the filtrate after the evaporation of the alcohol.

0.5 gm. of the air-dried preparation was gently ignited and the residue extracted with nitric acid. The filtrate showed no trace of chloride, but large amounts of calcium.

A neutral nuclein solution in ammonia was precipitated with calcium chloride and the precipitate washed and dried as above. 1 gm. of the preparation was dissolved in 25 cc. of a 1 per cent Na_2CO_3 solution, and the ammonia nitrogen determined according to Folin. Found: 0.9 mg. N.

When Precipitate A was stirred into water, the solution reacted neutral to litmus, and on addition of one drop of 0.1 N solution of sodium hydroxide to 10 cc. the reaction turned visibly alkaline.

From the filtrate of the calcium precipitate a precipitate was obtained with alcohol, which also behaved as a neutral calcium salt. This substance will be described in a second communication.

Neutral sodium and ammonium salts of the nuclein gave with $CaCl_2$ ($BaCl_2$ acted similarly) a double conversion, generating neutral calcium salts and sodium and ammonium chlorides, respectively.

Precipitate A dissolved easily in a solution of 1 per cent sodium hydroxide. Yet this solution showed slight biuret reaction. It was stirred several times in 2 per cent acetic acid and washed in the centrifuge. Large amounts of calcium acetate went into solution, but it was not possible to remove all the calcium in this way. When Precipitate A had been ex-

tracted three times with 2 per cent acetic acid, it nevertheless was dissolved completely in a solution of sodium hydroxide at neutral reaction. This solution was precipitated with a solution of $CaCl_2$, and the precipitate washed four times with 1 per cent $CaCl_2$ solution in the centrifuge. The precipitate gave now, dissolved in a solution of sodium hydroxide, no trace of biuret reaction. The calcium chloride was completely removed by washing with 96 per cent alcohol and the substance, after being treated with ether, was dried in the air. The preparation (A_1) was partly used for analysis and partly for further purification. From A_1—through extracting with acetic acid, dissolving with sodium hydroxide, and precipitating with calcium chloride (93 per cent of the nitrogen was precipitated)—another preparation (A_2) was obtained (precipitated in all four times with $CaCl_2$). One part of A_1 was treated with acetic acid, dissolved at neutral reaction with a solution of sodium hydroxide, and precipitated with two volumes of alcohol, which contained 0.2 per cent HCl. The precipitate was quickly centrifuged and washed twice with water and then several times with alcohol. After a treatment with ether it was dried in the air. The preparation (B_1) was partly used for analysis and partly further purified. By dissolving and precipitating twice with hydrochloric alcohol a preparation (B_2) was obtained.

The calcium salt of the nucleic acid was easily soluble in a solution of sodium chloride. One part of A_2 was stirred with a 10 per cent solution of sodium chloride. The solution was filtered from a very small dark-colored residue and then precipitated with four volumes of alcohol. The precipitate was washed many times with alcohol and then treated with ether and dried in the air. The white powder was dissolved in water and once more precipitated with alcohol, washed with 96 per cent alcohol and ether, and dried in the air. The preparation was called C.

Accordingly preparations were obtained as recorded in Table II.

50 gm. of nuclein were hydrolyzed with 5 per cent sulfuric acid for 5 hours at 100°C. The solution was filtered and the amount of sulfuric acid reduced with ammonia to 1 per cent. The solution was saturated while still hot with ammonium sulfate, and the precipitated, dark-colored albumoses were separated by filtration. The precipitate was dissolved in hot 1 per cent sulfuric acid, the solution saturated with ammonium sulfate, filtered, and the precipitate washed with a saturated solution of ammonium sulfate. The two filtrates were united.

(By direct experiments I have made sure that the purine bases in hydrolyzed albumin solution treated by this method remain quantitatively in the filtrates, and that after dilution with the same volume of water they are quantitatively precipitated with an ammoniacal silver solution.)

The filtrates were straw-colored. They were diluted with water, made strongly ammoniacal, and precipitated with an ammoniacal silver solution. The silver precipitate was put on a suction filter, washed, and the purine bases were then fractionated from it in the usual way.[2] The

[2] Steudel, H., in Abderhalden, E., Handbuch der biochemischen Arbeitsmethoden, Berlin and Vienna, 1910, ii, 585.

guanine was precipitated twice with ammonia, dissolved in a solution of sodium hydroxide, and precipitated with acetic acid. The adenine picrate was precipitated twice from the solution in sodium hydroxide by adding the calculated amount of hydrochloric acid. In the filtrate after the first precipitation of the adenine as picrate such a small amount of purine bases was left that xanthine and hypoxanthine could not be present. After being dried over sulfuric acid at room temperature the preparations were analyzed.

Guanine: 0.0476 gm. corresponded to 21.98 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl).

N: found 46.18 per cent; calculated 46.36 per cent.

Adenine-picrate: 0.3950 gm. was dissolved in alkali, the solution acidified with sulfuric acid, and the picric acid completely shaken out with ether. The ether was shaken with water and the nitrogen determined in tenths of the aqueous extracts. They corresponded to 7.60, 7.59, and 7.60 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl).

N: found 19.24 per cent; calculated for adenine, 19.24 per cent.

The preparation melted in 5 seconds at 280°C.

30 gm. of A_1 were hydrolyzed as above with 5 per cent sulfuric acid, and the solution was filtered and extracted with ether.

From the ether extract levulinic acid in small amounts could be isolated as silver salt.

0.1769 gm. gave 0.1130 gm. AgCl.

Ag: found 48.08 per cent; calculated 48.43 per cent.

The rest of the hydrolyzed solution was analyzed in the usual way for purine and pyrimidine bases. Guanine, adenine, and picrate of cytosine were isolated. The thymine fraction was lost. Yet, from the ether extracts typical thymine crystals were found (microscopic observation), and A_1 as well as B and C gave, when treated by Kossel and Neumann's method,[3] crystals that sublimed when heated, and which were soluble in alcohol and water.

Guanine (dissolved twice in a solution of sodium hydroxide, precipitated with acetic acid, and dried over sulfuric acid *in vacuo* at room temperature): 0.0261 gm. corresponded to 12.09 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl).

N: found 46.32 per cent; calculated 46.36 per cent.

Adenine picrate (once recrystallized and dried as above): from 0.4731 gm. the picric acid was removed and the nitrogen analyzed in tenths of the solution according to Kjeldahl. Two portions corresponded to 9.05 and 9.10 cc. N/14.01 $Na_2S_2O_3$ solution.

N: found 19.18 per cent; calculated for adenine 19.24 per cent.

The preparation melted in 5 seconds at 281°C.

Cytosine picrate (once recrystallized and dried as above): 0.1008 gm. gave 0.1295 gm. CO_2 and 0.0223 gm. H_2O (Dennstedt); 0.0469 gm. corresponded (after shaking out the picric acid) to 5.75 cc. N/14.01 $Na_2S_2O_3$ solution.

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[3] Kossel, A., and Neumann, A., *Ber. chem. Ges.*, 1893, xxvi, 2753.

	Found. *per cent*	Calculated. *per cent*
C	35.05	35.26
H	2.48	2.37
N	12.26	12.36*

* Calculated upon cytosine.

About 4 gm. of A_1 were dissolved in a solution of sodium hydroxide and the nitrogen was determined in a part of the solution. The total amount of N was 763.5 mg. The solution was neutralized and hydrolyzed for 5 hours with 5 per cent sulfuric acid at 100°C. After neutralization the solution was filtered and precipitated with an ammoniacal silver solution. The precipitate was washed and decomposed with hydrochloric acid. After a second precipitation and decomposition the hydrochloric acid filtrate was brought to dryness three times *in vacuo*, water being added after each concentration. The residue was dissolved in hot water, and the warm solution (150 cc.) was mixed with ammonia up to 3 per cent. After standing for 24 hours in a closed vessel the guanine was removed by filtration, washed with 30 cc. of ammonia, and dissolved in a solution of sodium hydroxide. The solution was made acid with sulfuric acid, and the guanine precipitated with ammoniacal silver solution (guanine fraction). Filtrate and washings from the precipitate of the guanine with 3 per cent ammonia (180 cc.) were precipitated with ammoniacal silver solution (adenine fraction).

The two silver precipitates were decomposed with hydrochloric acid, and two samples taken from each of the filtrates. From the guanine solution (300 cc.) 2 samples of 10 cc. were taken, and from the adenine solution (100 cc.) also 2 samples of 10 cc. The four samples were precipitated with ammoniacal silver solution, the precipitates washed with 1 per cent ammonia, suspended in water, and, after the addition of sodium carbonate, the ammonia nitrogen was completely removed according to Folin. The nitrogen in the residues was determined according to Kjeldahl. The samples of the guanine fraction corresponded to 13.15 and 13.19 cc., those of the adenine fraction to 14.72 cc. and 14.66 cc. N/14.01 $Na_2S_2O_3$ solution.

According to Wulff,[4] 0.015 gm. guanine is soluble in 100 cc. of 3 per cent ammonia. A corresponding correction for the guanine, which remained in the filtrate from the first guanine precipitate, was therefore necessary. The 180 cc. would, according to Wulff, contain 12.52 mg. of nitrogen as guanine. Out of the 763.5 mg. of nitrogen were then found 407.6 mg. as guanine and 134.4 mg. as adenine. The ratio $\frac{\text{guanine}}{\text{adenine}}$ was therefore $\frac{0.8792}{0.2592}$ = 3.39. The amounts of nitrogen (541 mg.) found in the purine

[4] Wulff, C., *Z. physiol. Chem.*, 1893, xvii, 505.

bases corresponded to about 71 per cent of the total amount of nitrogen (763.5 mg.). Of course, the values found for guanine and adenine cannot be regarded as exact. They give, however, an approximate idea of the proportion between guanine and adenine.

Guanine and adenine (according to analysis, respectively 46.20 and 51.86 per cent N, Kjeldahl) were each dissolved by sulfuric acid. The guanine solution contained in all 40.07 mg. of N and the adenine solution 29.0 mg. of N. The solutions were mixed and sulfuric acid was added up to 5 per cent. The mixture was heated 5 hours at 100°C. Then guanine and adenine were separated, as above, after precipitation as silver compounds.

TABLE I.

Time 2 × 24 hrs.

No.	Dry pancreas.	Fibrin.	Liquid.	Nitrogen in the filtrate.
	gm.	gm.	cc.	mg.
1	0.5	10	150 water.	51.6
2	0.5		150 0.06 N HCl.	8.5
3		10	150 0.06 N HCl.	9.8
4	0.5	10	150 0.06 N HCl.	19.1
5	0.5		150 0.06 N HCl.	23.0
6	0.5		150 0.06 N HCl.	21.0
7	0.5	10	150 water.	342.0

Found: 38.07 mg. guanine N and 26.14 mg. adenine N. $\frac{\text{Guanine}}{\text{Adenine}} = \frac{0.0864}{0.0504} = 1.71$. Calculated: $\frac{0.0864}{0.0559} = 1.55$. Losses = 7 per cent of total N.

A number of fractions of the purine bases were made in the different preparations. The course of the analysis was the same as described above. I also tried to determine the proportion between guanine and adenine in Hammarsten's β-protein and Umber's[5] protein. The silver compounds of the purine bases from these substances were precipitated from the hydrolyzed solutions, after saturation with ammonium sulfate.

[5] Umber, F., *Z. klin. Med.*, 1900, xl, 464.

TABLE II.

Preparation.	Precipitated with.	Color.	Solubility.	Precipitability.
Nuclein.	HCl twice.	White with a slight tendency to gray.	Nearly insoluble in water; easily soluble in alkali.	With acetic acid, HCl, and $CaCl_2$ in complete precipitations.
Neutral Ca salt.				
A_1	$CaCl_2$ twice.	White with a slight tendency to gray.	Nearly insoluble in water, 2 per cent acetic acid, and 0.2 per cent HCl; clearly soluble in 10 per cent NaCl solution.	
A_2	$CaCl_2$ four times.			
Free acid (contained some lime).				
B_1	HCl and alcohol once.	White with a slight tendency to gray.	Insoluble in water; easily soluble even at acid reaction in NaOH. 2 per cent solution straw-colored; a 10 per cent solution dark brown.	With acetic acid only at about 5 per cent opalescence in the solution. HCl and $CaCl_2$ solution precipitated nearly quantitatively.
B_2	HCl and alcohol thrice.			
Double salt of Ca and Na.				
C	Alcohol twice.	Quite white.	Easily and quite clearly soluble in water and 20 per cent sodium acetate. 5 per cent solution straw-colored.	Acetic acid precipitated only at 10 per cent acid in the solution; HCl and $CaCl_2$ solution precipitated nearly quantitatively.

The total amount of basic nitrogen was also determined directly. After hydrolysis, as described above, the silver compounds of the purine bases were precipitated, the precipitates washed, decomposed with HCl, the filtrates evaporated to dryness three times *in vacuo*, the residues dissolved in water once more, precipitated with silver from the ammoniacal solutions, the precipitates washed with 1 per cent ammonia, suspended in a solution of sodium carbonate, all the ammonia nitrogen was expelled, and the nitrogen determined in the residues according to Kjeldahl.

10 gm. of B_2 (p. 249) were (according to Steudel) added to 20 cc. of nitric acid at 0°C. and of a specific gravity of 1.02. After standing for 6 days at 0°C. the purine bases were fractioned in the usual way. The adenine was twice fractioned from guanine with ammonia. The guanine and the adenine picrate were once precipitated and weighed, without further cleaning after drying over sulfuric acid *in vacuo* at room temperature. Found: guanine, 1.2087 gm. (without correcting for the guanine, dissolved in the ammoniacal mother liquor); adenine picrate 1.0894 gm. (corresponding to 0.4041 gm. adenine).

A_1. About 4 gm. gave 407.6 mg. guanine N and 134.4 mg. adenine N; 0.8792 gm. guanine; 0.2592 gm. adenine.

1.6828 gm. (231.7 mg. total N) gave 133.1 mg. guanine N and 49.32 mg. adenine N. 17.06 per cent guanine N; 5.65 per cent adenine N; purine base N = 78.70 per cent of total N.

1.1754 gm. (161.9 mg. total N) gave 99.19 mg. guanine N and 31.94 mg. adenine N. 18.22 per cent guanine; 5.24 per cent adenine; purine base N = 81.0 per cent of total N.

0.9866 gm. (135.9 mg. total N) gave 84.34 mg. guanine N and 27.18 mg. adenine N. 18.44 per cent guanine; 5.31 per cent adenine; purine base N = 82.1 per cent of total N.

0.5584 gm. (76.89 mg. total N) gave 59.96 mg. purine base N = 78.0 per cent of total N.

0.8351 gm. (115.0 mg. total N) gave 92.75 mg. purine base N = 80.7 per cent of total N.

0.6590 gm. (90.7 mg. total N) gave 73.86 mg. purine base N = 81.4 per cent of total N.

0.4791 gm. (65.97 mg. total N) gave 49.15 mg. purine base N = 74.5 per cent of total N.

C. 1.6757 gm. (236.1 mg. total N) gave 127.4 mg. guanine N and 45.9 mg. adenine N. 16.40 per cent guanine; 5.28 per cent adenine; purine base N = 73.4 per cent of total N.

2.0481 gm. (288.6 mg. total N) gave 158.8 mg. guanine N and 56.3 mg. adenine N. 16.72 per cent guanine; 5.30 per cent adenine; purine base N = 74.5 per cent of total N.

A preparation of the β-protein gave 128.9 mg. guanine N and 44.4 mg. adenine N. Guanine = 0.2781 gm.; adenine = 0.0856 gm.; guanine: adenine = 3.25.

Umber's protein gave 217.1 mg. guanine N and 88.2 mg. adenine N. Guanine = 0.4683 gm.; adenine = 0.1702 gm.; guanine: adenine = 2.75.

In A_1 the loss in weight was determined by drying over P_2O_5 *in vacuo* at room temperature; after 2 weeks the weight was constant and remained unchanged for 6 months.

1.7928 gm. lost 0.2796 gm. = 15.45 per cent H_2O.
0.3678 " " 0.0571 " = 15.52 " " H_2O.
0.2926 " " 0.0456 " = 15.58 " " H_2O.
0.1840 " " 0.0281 " = 15.27 " " H_2O.

In the determination of the amounts of N, P, pentose, and Ca in the different preparations, the following values were found:

A_1. 0.1076 gm. corresponded to 14.81 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 13.76 per cent N.

0.0981 gm. corresponded to 13.52 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 13.78 per cent N.

0.0714 gm. corresponded to 9.83 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 13.77 per cent N.

0.2891 gm gave 0.0767 gm. $Mg_2P_2O_7$[6] = 7.40 per cent P.
0.2717 " " 0.0700 " $Mg_2P_2O_7$[6] = 7.18 " " P.
0.3106 " " 0.0820 " $Mg_2P_2O_7$[6] = 7.36 " " P.

2.5308 gm gave, after distillation with hydrochloric acid,[7] 0.5797 gm. furfurolphloroglucine = 23.17 per cent pentose.

0.4624 gm. of the dried substance gave (after melting with KOH, precipitating the phosphoric acid, according to the acetate method, and the calcium as oxalate) after igniting the calcium oxalate 0.0376 gm. CaO = 5.81 per cent Ca.

0.6464 gm. of the dried substance gave, treated in the same way, 0.0523 gm. CaO = 5.78 per cent Ca.

B_1. 0.0750 gm. corresponded to 11.19 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 14.92 per cent N.

0.0664 gm. corresponded to 9.91 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 14.92 per cent N.

0.2810 gm. gave 0.0791 gm. $Mg_2P_2O_7$ (Woy) = 7.85 per cent P.
0.2747 " " 0.0769 " $Mg_2P_2O_7$ " = 7.80 " " P.

B_2. 0.0694 gm. corresponded to 10.24 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 14.76 per cent N.

0.0843 gm. corresponded to 12.91 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 15.20 per cent N.

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[6] After melting with KOH and precipitating off the P, according to Woy, *Chem.-Ztg.*, 1897, xxi, 442, 469.

[7] Tollens, B., in Abderhalden E., Handbuch der biochemischen Arbeitsmethoden, Berlin and Vienna, 1910, ii, 130.

0.0876 gm. corresponded to 13.09 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 14.94 per cent N.
0.2382 gm. gave 0.0676 gm. $Mg_2P_2O_7$ (Woy) = 7.91 per cent P.
0.2771 " " 0.0792 " $Mg_2P_2O_7$ " = 7.97 " " P.
1.2080 gm. gave after distillation with hydrochloric acid, 0.3020 gm. furfurolphloroglucine = 25.50 per cent pentose.

C. 0.0892 gm. corresponded to 12.45 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 13.96 per cent N.
0.0711 gm. corresponded to 10.13 cc N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 14.25 per cent N.
0.2270 gm. gave 28.0 cc. N (at 739.0 mm. Hg and 20°C.) (Dumas) = 13.97 per cent N.
0.2998 gm. gave 37.1 cc. N (at 750.0 mm. Hg and 21°C.) (Dumas) = 14.18 per cent N.
0.2455 gm. gave 0.0675 gm. $Mg_2P_2O_7$ (Woy) = 7.66 per cent P.
0.3672 " " 0.0979 " $Mg_2P_2O_7$ " = 7.43 " " P.
0.3302 " " 0.0888 " $Mg_2P_2O_7$ " = 7.50 " " P.
1.1357 gm. gave after distillation with hydrochloric acid, 0.2867 gm. furfurolphloroglucine = 25.76 per cent pentose.

The results are summarized in Tables III and IV.

Before discussing the results, I would call attention to the fact that it is impossible to give even a probable formula for the analyzed nucleic acid. Nevertheless I venture to suggest a formula, only for the purpose of pointing out one possibility of composition and structure which approximately corresponds to the values found.

The relation between nitrogen and phosphorus was constant in the different preparations, a fact which strongly argues for a unit substance. As already mentioned the ratio $\frac{N}{P} = 1.89$ does not correspond either with that of a common tetranucleotide (1.69) or with that of a purine mononucleotide (2.26), while for a nucleic acid with 25 atoms of N and 6 atoms of P the ratio would be 1.88. The values found for the ratio $\frac{\text{guanine}}{\text{adenine}}$ argue for the probability that 1 molecule of the analyzed nucleic acid contains 3 molecules of guanine and 1 molecule of adenine (calculated $\frac{3 \text{ mols guanine}}{1 \text{ mol adenine}} = 3.36$).

About 75 per cent of the total amount of nitrogen was determined as purine base nitrogen. 3 molecules of guanine and 1

TABLE III.

Preparation.	N	P	H_2O	Ca determined on dried substance.	Guanine.	Adenine.	Purine base N in per cent of the total amount of N.	Pentose.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
A_1	13.76	7.40	15.45	5.78	17.06	5.65	71.0; 78.7	
	13.78	7.18	15.52		18.22	5.24	81.0; 82.1	
	13.77	7.36	15.58	5.81	18.44	5.31	78.0; 80.7	
			15.27				81.4; 74.5	
Average....	13.77	7.31	15.46	5.80	17.91	5.40	78.4	23.17
B_1	14.92	7.85						
	14.92	7.80						
Average....	14.92	7.83						
B_2	14.76	7.91						
	15.20	7.97						
	14.94							
Average....	14.96	7.94			12.09	4.04		25.50
C	13.96	7.66			16.40	5.28	73.4	
	14.25	7.43						
	13.97	7.50			16.72	5.30	74.5	
	14.18							
Average....	14.09	7.53			17.56	5.29	74.0	25.76

TABLE IV.

Preparation.	$\frac{N}{P}$	$\frac{\text{Guanine}}{\text{Adenine}}$	$\frac{\text{Pentose}}{N}$
A_1		3.02	
		3.48	
		3.47	
	1.88	3.32	1.68
B_1	1.90		
B_2	1.88	2.99	1.70
C		3.11	
		3.15	
	1.87	3.13	1.83
Average.....	1.89	3.15	1.74

molecule of adenine give together 20 atoms of N. With the assumption of 25 atoms of N in the examined nucleic acid, 5 atoms would stand in other groups. As cytosine and thymine were found, there is the possibility that the remaining 5 N atoms (20 per cent of the total nitrogen) could be divided between 1 molecule of thymine and 1 molecule of cytosine. The values thus far discussed could correspond to a compound of 2 molecules of guanylic acid and 1 molecule of tetranucleotide. With the assumption of the possible formulation for the "ideal" tetranucleotide, proposed by Steudel,[2] and more accurately by Levene and by Feulgen,[3] the following structure could be assumed.

TABLE V.

	Calculated.		On A_1, found values, counted to dry substance. Ca directly determined on dried substance.
	Formula I.	Formula II.	
	per cent	*per cent*	*per cent*
N	16.63	16.57	16.29
P	8.84	8.81	8.65
Ca	5.71	5.69	5.80
$\frac{\text{Pentose}}{\text{N}}$	0.857	1.71	1.68

2 molecules of guanylic acid united with 1 molecule of tetranucleotide by ester linkings between phosphoric acid and carbohydrate (Formula I), or a nucleic acid composed as the above mentioned, only with the difference that all the purine base nucleotides contain, as carbohydrate pentose and the pyrimidine base nucleotides, hexose (Formula II).

Values can accordingly be calculated for the 6-basic Ca salts as given in Table V.

The calculation is made with the following formulas: 6 mols phosphoric acid + $\begin{cases} 4 \text{ mols (I)} \\ 2 \text{ mols (II)} \end{cases}$ hexose ($C_6H_{10}O_4$) + $\begin{cases} 2 \text{ mols (I)} \\ 4 \text{ mols (II)} \end{cases}$ pentose ($C_5H_{10}O_5$) + 3 mols guanine + 1 mol adenine + 1 mol thymine + 1 mol cytosine + 3 atoms Ca. From this 17 mols water and 6 atoms hydrogen were deducted for the linkings. The calculated molecular weights were 2106.16 (I) and 2114.16 (II).

[3] Feulgen, R., *Z. physiol. Chem.*, 1918, ci, 288.

All the values found accord with both Formula I and Formula II except the values for the pentose, which explicitly speak in favor of Formula II. The method of furfurol distillation for the determination of pentose is, however, quite conventional, and furthermore different amounts of furfurol can be generated from hexose[9] during the distillation.

Once more, I want to point out that I am quite conscious of the fact that the above speculation about the formulas lacks adequate experimental basis.

As mentioned several times before, Preparation C gives a flocky precipitate when it is warmed in alkaline solution and neutralized with acetic acid. This precipitate was at first noted as a dense opalescence, and then became at a slightly alkaline reaction a gelatinous porridge. After standing for 24 hours in the cold the precipitate was filtered, washed several times with cold water, dissolved in a solution of sodium hydroxide, and the solution neutralized with acetic acid. After 24 hours it was filtered and washed with cold water, then dissolved in water, precipitated with two volumes of alcohol, and dried with alcohol and ether.

In this preparation (dried in the air) the N and P analyses were made.

0.0492 gm. corresponded to 6.96 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 14.15 per cent N.

0.0586 gm. corresponded to 8.41 cc. N/14.01 $Na_2S_2O_3$ solution (Kjeldahl) = 14.35 per cent N.

0.2647 gm. gave 0.0626 gm. $Mg_2P_2O_7$ (Woy) = 6.59 per cent P.

0.4568 " " 0.1087 " $Mg_2P_2O_7$ " = 6.63 " " P.

$$\frac{N}{P} = \frac{14.25}{6.61} = 2.16.$$ For guanylic acid calculated to 2.26.

0.5 gm. of the preparation was decomposed with nitric acid according to Steudel. Guanine could be precipitated with ammonia in ample quantities. In the ammoniacal filtrate only traces of purine bases remained. The substance is apparently composed of an alkali salt of guanylic acid. The preparation was easily soluble in water, and the aqueous solution gave with a small amount of sodium acetate a gelatinous precipitate. By adding acetic acid it was turned into a soft jelly. With calcium chloride the aqueous solution gave at once a solid jelly.

[9] Steudel, R., *Z. physiol. Chem.*, 1908, lvi, 212.

These circumstances accord with the observations of Feulgen[10] on the solubility of the alkali salts of guanylic acid. According to this investigator the neutral alkali salt of guanylic acid is easily soluble in water and is not precipitated by acetic acid but transformed into the gelatinous acid salt. Furthermore the alkali salts of guanylic acid are dissolved with great difficulty in a solution of sodium acetate, a fact on which Feulgen has based a method for isolating guanylic acid. The low solubility in sodium acetate is without doubt the reason why the alkali salt of the guanylic acid was precipitated by neutralizing with acetic acid the alkaline hydrolyzed solution.

The first filtrate from the alkali salt of guanylic acid still contained guanylic acid, which could be (according to Feulgen) precipitated with sodium acetate.

The results of the experiments on the nucleic acids, which remained in the solution, will be reported in a second communication.

The reaction on warming with 1 per cent solution of sodium hydroxide was followed by observations of the alterations in the hydrogen ion concentration, resistance, and freezing point.

In a 4 per cent solution of Preparation C in a 1 per cent solution of sodium hydroxide [H], resistance,[11] and Δ[11] were determined before and after warming. The solutions were prepared just before the experiments and then, during the experiments, were protected by tubes of soda-lime from the carbonic acid in the air.

In each experiment 25 cc. were warmed during 10 minutes one or several times in a boiling water bath. The solution remained absolutely clear even after warming 30 minutes. For the determination of [H] and resistance the same solution was used. The transfer of the solution from the heating vessel to the measuring vessel for the electric resistance, and from this to the hydrogen electrode, was made with protection against the carbonic acid of the air. The determination of the freezing point could not be made in a solution of 1 per cent sodium hydroxide, because precipitation occurred at the low temperature in spite of the alkaline

[10] Feulgen, R., *Z. physiol. Chem.*, 1919, cvi, 249.

[11] The directly found values of resistance in ohms and the freezing point in degrees, without consideration of the resistance capacity of the measuring vessel or of the zero correction of the thermometer.

reaction. These determinations were accordingly made with 4 per cent solution of Preparation C in a solution of 2 per cent sodium hydroxide. During the heating, nevertheless, a very small dark-colored precipitate formed, which, it may be, makes the results of these determinations somewhat uncertain.

The values for resistance and Δ are the directly observed values without any consideration of the calibration of the instruments, as only the relative values here are of interest (Table VI).

TABLE VI.

Preparation C, a 4 per cent* solution in a solution of 1 per cent NaOH.	Resistance.	[H] normality.	Δ†
	ohms		*C.*
Before heating	173	8.20^{-14}	2.468
After standing 24 hrs. at 0°	173	8.20^{-14}	
" warming 10 min	217	1.81^{-13}	2.469
" " 20 "	217.9	2.21^{-13}	2.460
" " 30 "	220	2.29^{-13}	

* As about 0.001 molecular, calculated as nucleic acid.

† 2 per cent solution of NaOH.

TABLE VII.

Alkali salt of guanylic acid, 4 per cent solution in 1 per cent solution of NaOH.	Resistance.	[H]
	ohms	
Before heating	147	1.31^{-13}
After " 10 min	149	1.31^{-13}

Different experiments, even with Preparation B_2, were made with quite the same results.

The alkali salt of guanylic acid (compare above) was dissolved in a solution of 1 per cent sodium hydroxide and resistance and [H] were determined before and after warming (Table VII).

The experiments (Table VI) show that [H] was increased through heating of the alkaline solution, which is the same as a disappearance of OH ions. The increase in the resistance may thus be ascribed to the disappearance of mobile OH ions. The freezing point was not altered by heating, a fact which forces the conclusion that the absolute quantity of some other kind of

ions has increased. All the found values for resistance, [H], and Δ showed thus in good agreement that an hydrolysis had taken place, whereby 2 or more molecules had been separated from each other.

By repeated experiments I have convinced myself that no phosphoric acid was detached during the heating with sodium hydroxide. On the other hand it was easy to show that by heating a solution of C in water ample quantities of phosphoric acid were detached, and that this cleavage could be prevented by small amounts of sodium hydroxide.

The cleavage of phosphoric acid from nucleic acids by heating their alkaline solutions proceeds gradually, as Feulgen[12] has shown. As is shown in Table VI, the hydrolysis, however, was finished after 10 minutes (probably much earlier). The same is shown by Table VII. Here the alkali salt of guanylic acid, which had been isolated from the hydrolyzed solution, was heated in a solution of 1 per cent sodium hydroxide, without any alteration of the hydrogen ion concentration, or of the electrical resistance.

It is probable that, through the hydrolysis in alkaline solution, ester linkings between two or more nucleic acids (of which one is guanylic acid) were dissolved.

The substance, which was prepared by Feulgen[1] by enzymatic digestion of β-protein from pancreas, and by him called "Guanylnucleinsäure" is without any doubt closely related to the one described by me. Feulgen found the same cleavage products for his nucleic acid that I have found. After hydrolysis it was possible for him to precipitate an alkali salt of guanylic acid with sodium acetate. From the amounts of guanylic acid found he calculated that 1 molecule of guanylic acid is united with 1 molecule of tetranucleotide. According to my results, probably at least 2 molecules of guanylic acid are to be found in every molecule of my nucleic acid. Feulgen's nucleic acid was dextrorotatory, which was also the case with my nucleic acid.

Of greater interest than the settling of the quantitative composition of these complicated substances seems to me to be the proof of the occurrence of genuine compounds, capable of being hydrolytically split, between "single" and "compound" nucleic acids.

[12] Feulgen, R., *Z. physiol. Chem.*, 1914, xci, 165.

I suggest "conjugated nucleic acids" as a name for such compounds. Whether "conjugated nucleic acids" occur in the organisms as such, or whether they are only artificial products, is a question outside the province of this paper.

The experiments are being continued.

WATER-SOLUBLE VITAMINES.

I. ARE THE ANTINEURITIC AND THE GROWTH-PROMOTING WATER-SOLUBLE B VITAMINES THE SAME?

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The object of the present paper is to present data which we believe indicate that a more definite distinction should be made, for the present at least, between the antineuritic and certain of the other water-soluble vitamines, particularly those that relate to the growth stimuli.

Since making our preliminary reports (1, 2) on this subject, Mitchell (3) has presented an excellent review of the literature, and we will limit ourselves to a brief summary of the published work that bears directly upon the particular phase of the problem as we have approached it; namely, the stability of the water-soluble vitamines to heat.

We have, therefore, made a compilation of the available data on the basis of the particular biological test for which the trials were planned with only a secondary consideration of the descriptive names associated with the vitamine employed.

In comparing the data in Tables I and II, it should be borne in mind, as Chick and Hume (4) have brought out, that the amount of ration consumed may carry an excess of the vitamine beyond the minimum requirements, and, if so, a partial destruction of the vitamine by heat, alkali, or other causes might not necessarily become evident in the biological tests.

Comparing the results in the two tables, they tend to indicate that the antineuritic and the growth-promoting vitamines, as measured by polyneuritic pigeons and young rats respectively, were fairly stable at temperatures around 100–105°C. At higher temperatures, the antineuritic vitamine seemed to be less stable

TABLE I.

*Polyneuritis (Antineuritic Vitamine).**

Series.	Temperature and time of heating.	Destruction.	Reference.
	Section I. Fowl: pigeon, chicken, and duck.		
A	100°C., moist heat.		
1a†	Egg yolk, 4 min.	None apparent.	Cooper (5).
2a	" " in presence of dilute alkali.	Total.	Steenbock (6).
3b†	Buffalo meat, several days.	"	Grijns (7).
4b	Beef, 30 min.	None apparent.	Holst (8).
5a	Yeast extract, 1 hr.	Slight.	Chick and Hume (9).
6a	Wheat embryo, 1 hr.	Very slight.	Chick and Hume (9, 10).
7b	Unmilled rice, 3 hrs.	None apparent.	Eykman (11).
B	110°, autoclave, 30 min.		
8b	Beef.	Appreciable.	Holst (8).
C	113°, autoclave, 1 hr.		
9a	Wheat embryo (102–107° for 40 min.).‡	Slight.	Chick and Hume (10).
D	115°, autoclave, 2 hrs.		
10b	Unmilled rice, millet, oats, rye, barley.	Total.	Eykman (11).
E	120°, 15 pounds pressure, 30 min.		
11b	Beef, eggs.	Marked.	Vedder (12).
12b	Dried peas, unhulled barley.	None apparent.	Holst (8).
F	120°, 15 pounds pressure, 1 hr.		
13b	Beef.	Total.	"
G	120°, 15 pounds pressure, 1½ hrs.		
14b	Unmilled rice.	"	Weil, Mouriquand, and Michel (13).
15b	Barley.	"	Weil and Mouriquand (14).

TABLE I—*Concluded.*

Series.	Temperature and time of heating.	Destruction.	Reference.
	Section I. Fowl: pigeon, chicken, and duck—*Concluded.*		
H	120°, 15 pounds pressure, 2 hrs.		
16b	Unmilled rice, Katjidgo beans, buffalo meat.	Total.	Grijns (7), Eykman (11).
17b	Horse meat.	None apparent.	Eykman (11).
18b	Rye, unmilled rice, millet, oats, barley.	Total.	Holst (8).
I	122°, autoclave, 1 hr.		
19a	Yeast extract and wheat embryo, (110–117° for 40 min.).‡	Appreciable.	Chick and Hume (9, 10).
J	122°, autoclave, 2½ hrs.		
20a	Yeast extract and wheat embryo (118–124° for 2 hrs.)‡	Very marked.	" "
K	125°, autoclave, 2 hrs.		
21b	Unmilled rice and millet.	Total.	Eykman (11).
L	135°, autoclave, 2 hrs.		
22b	Unmilled rice, rye, millet, oats, barley.	"	Holst (8).
	Section II. Dogs.		
M	120–130°, autoclave, 1 to 3 hrs.		
23b	Horse meat.	Total.	Schaumann (15).
24b	Lean beef in presence of 10 per cent Na_2CO_3.	"	Voegtlin and Lake (16).
	Section III. Cats.		
N	120°, 15 pounds pressure, 3 hrs.		
25b	Lean beef.	Appreciable.	Voegtlin and Lake (16).
26b	" " in presence of 10 per cent Na_2CO_3.	Total.	" "

* Designated by some as water-soluble B, growth-promoting factor, neuritic-preventing vitamine, and antiberi-beri vitamine.

† a, tested curatively by giving it to polyneuritic fowl; b, tested prophylactically by feeding normal fowl.

‡ Temperature of the substance itself and length of time it remained at this point.

TABLE II.

Growth-Promoting Vitamine (Water-Soluble B).*

Series.	Temperature and time of heating.	Destruction.	Reference.
	Section I. Rats.		
A	90–100°C., dry heat, several hrs.		
1	Liver, heart, kidney, brain.	None apparent.	Osborne and Mendel (17).
B	100°, moist heat.		
2	Protein-free milk, 2 min.	None apparent.	Osborne amd Mendel (18).
3	Milk whey, 6 hrs.	" "	McCollum and Davis (19).
4	Extract yeast, 30 min.	" "	Drummond (20).
5	Extract wheat embryo, in presence of 0.28 per cent NaOH, 1 hr.	Total.	McCollum and Simmonds (21).
6	Extract yeast, in presence of 5 per cent NaOH, 5 hrs.	Marked.	Drummond (20).
7	Soy beans, navy beans, cabbage, 40 to 120 min.	None apparent.	Daniels and McClurg (22).
8	Soy beans (120 min.), navy beans (90 min.), cabbage (45 min.), in presence of 5 per cent $NaHCO_3$.	" "	" "
9	Carrots.†	" "	Denton and Kohman (23).
10	Yeast, 0.1 N NaOH for 21.5 hrs. in cold. 2 hrs. heating.	" "	Osborne, Wakeman, and Ferry (24).
C	105°, dry heat, several hrs.		
11	Meat powder (lean beef).	" "	Osborne and Mendel (25).
12	Beef extract.	" "	" "
13	Compressed yeast.	" "	Hawk, Fishback, and Bergeim (26).

* Designated by some as water-soluble B, antineuritic vitamine, and water-soluble growth-promoting accessory factor.

† Placed in cans, then immersed in water, and heated at 100° for 2 hrs.

TABLE II—*Continued.*

Series.	Temperature and time of heating.	Destruction.	Reference.
	Section I. Rats—*Continued.*		
D	120°, 15 pounds pressure, 20 min.		
14	Navy beans, cabbage.	None apparent.	Daniels and McClurg (22).
15	Extract, navy beans.	" "	" "
E	120°, 15 pounds pressure, 30 min.		
16	Extract yeast.	Marked.	Drummond (20).
17	Soy bean flour.	None apparent.	Cohen and Mendel (27).
18	Extract, navy and soy beans, in presence of 0.1 N NaOH.	" "	Daniels and McClurg (22).
F	120°, 15 pounds pressure, 40 min.		
19	Navy beans.	None apparent.	McCollum and Simmonds (21).
20	Soy beans.	" "	Daniels and McClurg (22).
21	Extract, soy beans.	" "	" "
G	120°, 15 pounds pressure, 1 hr.		
22	Wheat embryo, milk whey.	" "	McCollum and Davis (19).
23	Extract, navy bean, in presence of 0.1 N NaOH.	" "	Daniels and McClurg (22).
H	120°, 15 pounds pressure, 1¼ hrs.		
24	Navy bean.	" "	McCollum, Simmonds, and Pitz (28).

TABLE II—*Concluded.*

Series.	Temperature and time of heating.	Destruction	Reference.
	Section I. Rats—*Concluded.*		
I 25	120°, 15 pounds pressure, 3 hrs. Lean beef, in presence of 10 per cent Na_2CO_3.	None apparent.	Voegtlin and Lake (16).
	Section II. Yeast cell.		
J 26	120°, 15 pounds pressure, 30 min. Yeast extract.	Slight.	Williams (29).

to heat and alkali than the rat growth-promoting vitamine, as shown in the case of wheat embryo (Table I, Series I, and Table II, Series G) and in lean beef (Table I, Series M and N, and Table II, Series I). The fact that no data were given as to the amount of food consumed by the rats, dogs, and cats in Voegtlin and Lake's work (16) is unfortunate as one might then have been able to evaluate their data from the standpoints under consideration here.

EXPERIMENTAL.

The plan of the series of trials reported was to use the *same* source of water-soluble vitamines for the studies on polyneuritis in pigeons and the rate of growth in young rats.

The basal food employed was unmilled rice. This furnished the only source of the water-soluble antineuritic and growth-promoting vitamines. In the case of the pigeons, the unground rice constituted the sole food. Gravel was offered once a week. With the rats, the ground rice was so supplemented with lactalbumin, salt mixture (21), butter fat, and lard that it formed a balanced ration for growth. The fundamental difference in the diets of the respective groups of pigeons and rats was in the use of unmilled rice that was unheated for the controls and of rices that were heated at different temperatures.

The heating of the rice was carried out as follows: in the air oven at 120°C. for 2 hours, after the temperature reached this point; and in the autoclave at 120° and 15 pounds pressure for

1, 2, and 6 hours, respectively—making in all four different samples. In some of the trials the pigeons were allowed to eat their food at will, others were force-fed as soon as they began to refuse their food. The pigeons were put in cages in groups of seven each. Each rat was kept in a separate compartment. The food intake of the rats was determined directly, that for the pigeons had to be calculated from the total consumption of rice for the entire group.

DISCUSSION.

Part I. Influence of Degree of Heating on the Antineuritic Vitamine, Using Pigeons.

The results presented in Charts 1 and 2 show the relative rate at which pigeons come down with polyneuritis when fed *ad libitum* unheated and heated unmilled rice. From these it is evident that some decided change took place in the rices that where subjected to the longer periods of heating. Naturally the first question that arises is in regard to the amount of the food consumed by the different lots. A fair idea of this is given in Table III.

The values are somewhat approximate due to the fact that the pigeons were fed in groups and also that they scattered some food. The refused food in the cups and on the metal floor of the cages was collected and subtracted from the total amounts offered.

TABLE III.

Food Consumed by Pigeons Fed ad Libitum.

(Gm. of food per day per 100 gm. of body weight.)

Period.	Unheated rice.	Rice, 1 hr. in autoclave at 120°.	Rice, 2 hrs. in autoclave at 120°.	Rice, 6 hrs. in autoclave at 120°.	Rice, 2 hrs. dry heat at 120°.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
1st week.....	7.6	8.1	8.0	7.2	8.8
2nd "	8.0	8.6	6.3	5.8	6.3
4th "	8.2	9.0	4.9	4.8	6.5
Average.....	8.0	8.6	6.4	5.9	6.7

The results indicate that the loss in weight varied inversely with the amount of rice eaten.

The fact that the food intake varied in this manner suggested that two or three factors might be involved in the causation of the onset of polyneuritis. The excessive heating might have destroyed the antineuritic vitamine; it might have produced certain toxic substances which prevented the vitamine from being potent; or it might have altered the physical condition of the rice making it harder and less easily digestible.

In view of these possibilities, it was decided to force-feed pigeons and compare the effects produced with those fed *ad libitum*. These curves are presented in Chart 3. In the main the results obtained were identical with what was found when the pigeons were fed *ad libitum*.

The question of the formation of toxic substances is still unanswered. In view of this, it was decided to carry out a series of experiments by force-feeding pigeons on unheated *milled* rice and then treating them with small quantities of an extract of autolyzed yeast which had been heated in the same manner as the natural or unmilled rices in Chart 3. In this way, it would be possible to eliminate any resultant conditions produced in the unmilled rice during the heating, such as undue hardness, peculiar aroma, etc. Since the amount of the vitamine extract given in this treatment was relatively small, being 0.19 gm. daily, it would seem that the toxic effect, if any, that could have been caused by adding this material to the rice diet was too insignificant to be considered seriously. The results are given in the first half of Chart 9.

In order to test this point of toxicity further, curative trials were made with extracts containing the antineuritic vitamine both before and after heating. Besides, vitamine extracts were treated with fullers' earth according to the method of Seidell (30) and the activated silicate was used. The results are presented in Table IV. These results together with those in Chart 9 show conclusively, even if reasonable allowances are made for the indefiniteness that sometimes appears in cases of polyneuritic pigeons, that toxicity was apparently a minor factor and that the antineuritic vitamine was totally destroyed by heating for 2 and 6 hours in the autoclave at 120° and 15 pounds pressure.

TABLE IV.

Treatment of Typical Polyneuritic Pigeons with Heated and Unheated Vitamine Preparations.

Bird No.	Treatment.	Response to treatment.
331	0.3 gm. unheated yeast extract.	Good recovery.
326	0.3 " " " "	" "
338	0.1 " " " "	Improved.
300	0.5 " 6 hrs. autoclaved yeast extract in a.m.	No better. Given a second treatment in p.m.
	0.5 " 6 hrs. autoclaved yeast extract in p.m.	Found dead next morning.
293	0.5 " 6 hrs. autoclaved yeast extract in a.m.	No better. Given a second treatment in p.m.
	0.5 " 2 hrs. autoclaved yeast extract in p.m.	Found dead next morning.
303	0.5 " 6 hrs. autoclaved yeast extract.	" " " "
394	0.5 " 2 hrs. autoclaved yeast extract.	" " " "
332	0.5 " 2 hrs. autoclaved yeast extract.	" " " "
405	0.5 " 2 hrs. yeast extract, 1st day.	No better. Treated following morning. Good recovery.
	0.5 " unheated yeast extract, 2nd day.	
414	0.6 " unheated activated protein-free milk.	Very good recovery.
436	0.6 " unheated activated protein-free milk.	" " "
433	0.6 " activated 6 hrs. autoclaved yeast extract.	No better. Treated second day.
	0.6 " activated unheated yeast extract, 2nd day.	Very good recovery.
422	0.6 " activated unheated yeast extract.	Good recovery.
404	0.6 " 6 hrs. autoclaved activated protein-free milk.	Found dead next morning.
432	0.6 " 6 hrs. autoclaved activated protein-free milk, 1st day.	No better. Treated second day.
	0.6 " unheated activated protein-free milk, 2nd day.	Very good recovery.

Part II. Influence of Degree of Heating on the Water-Soluble B Growth-Promoting Vitamine, in Rats.

Four groups of young rats were so fed that a comparison of the rate of growth of each would indicate the relative degree of destruction of this water-soluble vitamine by heating the rice. The ration for the control group was made up as follows:

		per cent
Period 1.	Unmilled rice, unheated	64.0
	Lactalbumin	6.0
	Butter fat	18.0
	Lard	10.0
	Salt mixture	2.0
Period 2.	Unmilled rice, unheated	89.7
	Lactalbumin	3.3
	Butter fat	5.0
	Salt mixture	2.0

Equivalent percentages of rice were used throughout, calculated on the dry basis, and then the necessary amount of water was added to bring all the rations to the same moisture content. We recognize that this method of adjusting the rations did not necessarily allow for the variations in palatability that might have been brought about during the heating of the rices. The food intake of each rat should, however, assist in interpreting the results from this standpoint.

The data are presented in Charts 4, 5, 6, and 7. When the individuals in the groups are compared, the greatest variation is found in the case of the 6 hours autoclaved rice (Chart 7).

In Chart 8 the average results for each group are given. These average group curves indicate that the heating processes apparently had some detrimental effect during the 6 hours in the autoclave at 120° and but very slight, if any, effect during 2 hours at 120° either in the oven or autoclave. A study of these data in connection with the food intake and the percentage gain per gm. of food consumed (Tables V and VI) will assist further in evaluating the rations.

In Table V, the amount of food consumed per day per rat is given. The differences in food intake between Groups 40, 50, and 60 were no greater than the differences between the values for the individual rats in the respective groups. In the case of

Group 70, however, the rats ate less of the ration, except Rat 75 which consumed as much as some of the animals in the other groups. The gains in weight of this rat compared favorably with those in the other groups having an equivalent food intake.

TABLE V.

Food Consumed.

Group No.	Ration and rat No.	Food consumed per day per rat.				
		Rat 1.	Rat 2.	Rat 3.	Rat 4.	Group average.
	Period 1 (75 days).					
		gm.	*gm.*	*gm.*	*gm.*	*gm.*
40	Unheated rice. Rats 41, 42, 43.	5.9	5.6	4.2		5.2
50	2 hrs. dry heat 120°. Rats 52, 54, 56.	4.9	6.5	5.9		5.8
60	2 hrs. autoclave 120°. Rats 62, 63, 64, 66.	5.6	4.5	4.2	5.2	4.9
70	6 hrs. autoclave 120°. Rats 71, 72, 73, 75.	3.3	2.6	3.1	4.3	3.3
	Period 2 (50 days).					
40	Unheated rice. Rats 41, 42, 43.	10.8	10.1	11.5		10.8
50	2 hrs. dry heat 120°. Rats 52, 54, 56.	8.2	10.1	11.3		9.9
60	2 hrs. autoclave 120°. Rats 62, 63, 64, 66.	7.0	8.1	7.8	9.0	8.0
70	6 hrs. autoclave 120°. Rats 71, 72, 73, 75.	4.7	3.3	3.6	7.5	4.8
	Periods 1 and 2 (125 days).					
40	Unheated rice. Rats 41, 42, 43.	7.8	7.4	7.1		7.4
50	2 hrs. dry heat 120°. Rats 52, 54, 56.	6.2	7.9	8.0		7.4
60	2 hrs. autoclave 120°. Rats 62, 63, 64, 66.	6.1	5.9	5.6	6.8	6.1
70	6 hrs. autoclave 120°. Rats 71, 72, 73, 75.	3.9	3.2	3.7	5.8	4.1
	Period 3 (21 days).					
70	Unheated rice (same as No. 40). Rats 71, 72, 73, 75.	9.0	5.5	7.7	9.7	8.0

TABLE VI

Gain per Gm. of Food Consumed.

Group No.	Ration and rat No.	Gain per gm. of food.				
		Rat 1.	Rat 2.	Rat 3.	Rat 4.	Group average.
	Period 1 (75 days).					
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
40	Unheated rice. Rats 41, 42, 43.	20.3	20.0	17.5		19.3
50	2 hrs. dry heat 120°. Rats 52, 54, 56.	20.9	17.7	22.2		20.3
60	2 hrs. autoclave 120°. Rats 62, 63, 64, 66.	18.2	20.0	14.5	16.1	17.2
70	6 hrs. autoclave 120°. Rats 71, 72, 73, 75.	15.1	(6.9)	13.0	16.6	14.9
	Period 2 (50 days).					
40	Unheated rice. Rats 41, 42, 43.	8.8	7.7	11.1		9.2
50	2 hrs. dry heat 120°. Rats 52, 54, 56.	8.3	4.0	11.4		7.9
60	2 hrs. autoclave 120°. Rats 62, 63, 64, 66.	6.7	7.1	12.6	12.7	9.8
70	6 hrs. autoclave 120°. Rats 71, 72, 73, 75.	10.6	(3.3)	3.5	11.5	8.5
	Periods 1 and 2 (125 days).					
40	Unheated rice. Rats 41, 42, 43.	14.5	13.8	14.3		14.2
50	2 hrs. dry heat 120°. Rats 52, 54, 56.	14.6	10.8	16.8		14.1
60	2 hrs. autoclave 120°. Rats 62, 63, 64, 66.	12.4	13.5	13.5	14.4	13.4
70	6 hrs. autoclave 120°. Rats 71, 72, 73, 75.	12.5	(5.1)	8.2	14.0	11.6
	Period 3 (21 days).					
70	Unheated rice (same as No. 40). Rats 71, 72, 73, 75.	19.0	(19.8)	18.0	24.1	20.4

If the growth-promoting water-soluble vitamine had been destroyed, the rate of growth for Rat 75 would have been much less. We are confronted, therefore, with the question of the palatability of the ration and as to what the other rats in the group would have gained if they had eaten as much food. This

point is further evidenced in Period 3 where all the rats ate more and made better gains than in Periods 1 and 2. Osborne and Mendel (31) found similar conditions in palatability in the case of soy bean.

When the rations (Table VI) on the basis of the gain made per gm. of food consumed are compared, it is evident that the diets for Groups 40, 50, and 60 were all practically of equal value. The data for Group 70, Period 1, compare favorably with the lower values for these groups. In Period 2, two of the rats did very well while the other two did very poorly. For the entire time, Rats 71 and 75 were able to utilize their food to as much advantage as the rats in the other three groups, showing that this ration was as economical as the others.

It is evident from Period 3 that all the rats in this group were capable of making good gains. Therefore, if their food consumption in Periods 1 and 2 had been equal to that of the rats in Group 40 and they had maintained the same degree of utilization of their food that they did in the test trial, the gains would have been larger than any of the other groups.

As another means of determining the effect of heat on the water-soluble growth-promoting vitamine, some rats were placed on a ration that lacked this vitamine and when they showed definite signs of retardation in growth 1 per cent of an extract of brewer's yeast, after being heated in the autoclave for 6 hours at 120°, was added. The diet was made up as follows:

	per cent
Lactalbumin	11.3
Starch	32.7
Lactose (purified)	24.6
Salt mixture	3.4
Butter fat	13.0
Lard	15.0

The curves are given in the second half of Chart 9.

SUMMARY.

In the foregoing discussion we have shown by comparing the antineuritic (pigeon) and the water-soluble B growth-promoting (rat) vitamines that in the former case the vitamine was altered

by heating the food or extracts containing it to certain temperatures while the other vitamine, obtained from the same food and extracts, apparently remained potent under the same conditions of heating. This is perhaps illustrated most definitely in Chart 9 where pigeons came down with polyneuritis on the one hand and young rats grew on the other.

The authors would call attention to the fact that the minimum vitamine requirement of the rats may have been lower per gm. of body weight than that of the pigeons. This latter point was intended to be covered in part when the amount of unmilled rice in the rat rations was increased from 64.0 to 89.7 per cent. For, first, if the heated rices were toxic the rate of growth would be definitely lowered by feeding larger amounts of rice, and, second, if the supply of the growth-promoting vitamine was sufficiently low in the 64.0 per cent ration a slight or partial destruction of the vitamine by heat would be apparent. And upon increasing the amount of rice, the quantity of vitamine would be increased and there would then be an added increment of growth.

The fact that the direction of the curves was not altered to any appreciable extent when this change was made suggests that the heated rices were not toxic, and, since the amount of water-soluble growth-promoting vitamine in the 64.0 per cent ration was apparently above the minimum requirements, these data are none too definite as to a partial destruction of the vitamine. However, it would appear if the antineuritic vitamine was the same as the water-soluble B that in the treatment of pigeons with increasing doses of heated extracts and in the force-feeding of them with the heated rices there should have been some response if the vitamine was but slightly or partially destroyed. In order to answer this question definitely, it will be necessary to measure quantitatively the amount of vitamine consumed.

CONCLUSIONS.

1. The antineuritic vitamine (pigeons) in unmilled rice is stable to heat at 120°C. and 15 pounds pressure for 1 hour. It is partially altered by heating in the air oven at 120° for 2 hours, and totally destroyed at 120° and 15 pounds pressure in 2 and 6 hours. The vitamine in extracts is more easily altered by heat.

2. The water-soluble B vitamine (rats) in unmilled rice appears to be stable to heat at these same temperatures, that is, it is not distinctly or totally broken down. Whether this vitamine was slightly destroyed could not be definitely ascertained due to the lack of quantitative methods.

3. These findings suggest tentatively, at least, that the antineuritic (pigeons) and the water-soluble B (rats) vitamines are not the same, and that it would be better to consider them as being different until there is further proof to the contrary.

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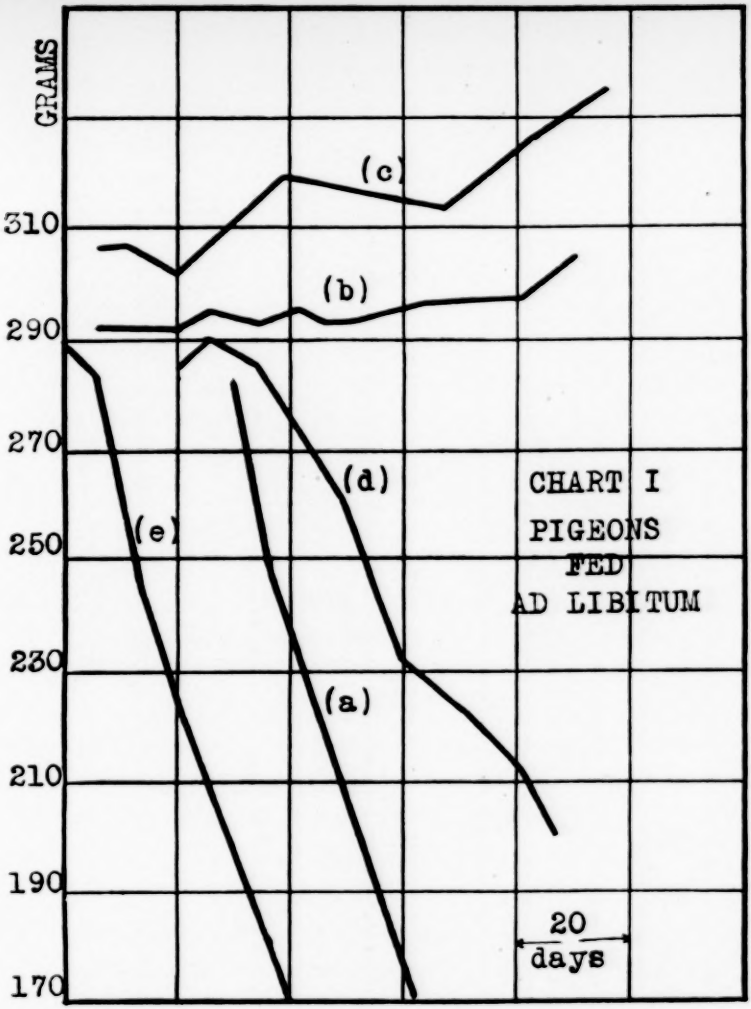

CHART 1. Represents the average body weight of the different groups that were fed *ad libitum*. Curve (a) for the milled rice; (b) for the unheated unmilled rice; (c) for the unmilled rice heated in the autoclave at 120°C. for 1 hr.; and (d) and (e) for the 2 and 6 hrs. autoclaved rices, respectively.

The heating for 1 hr. in the autoclave (Curve (c)) had no detrimental effect on the antineuritic vitamine. In the case of the 2 and 6 hrs. autoclaved rices, the losses in weight were very marked, being practically the same as for the milled rice (Curve (a)).

The pigeons on the 6 hrs. heated rice refused their feed and regurgitated part of it sooner than the ones that were fed on the 2 hrs. autoclaved rice. The general symptom complex of these two groups of pigeons was very similar to that observed in the feeding of milled rice.

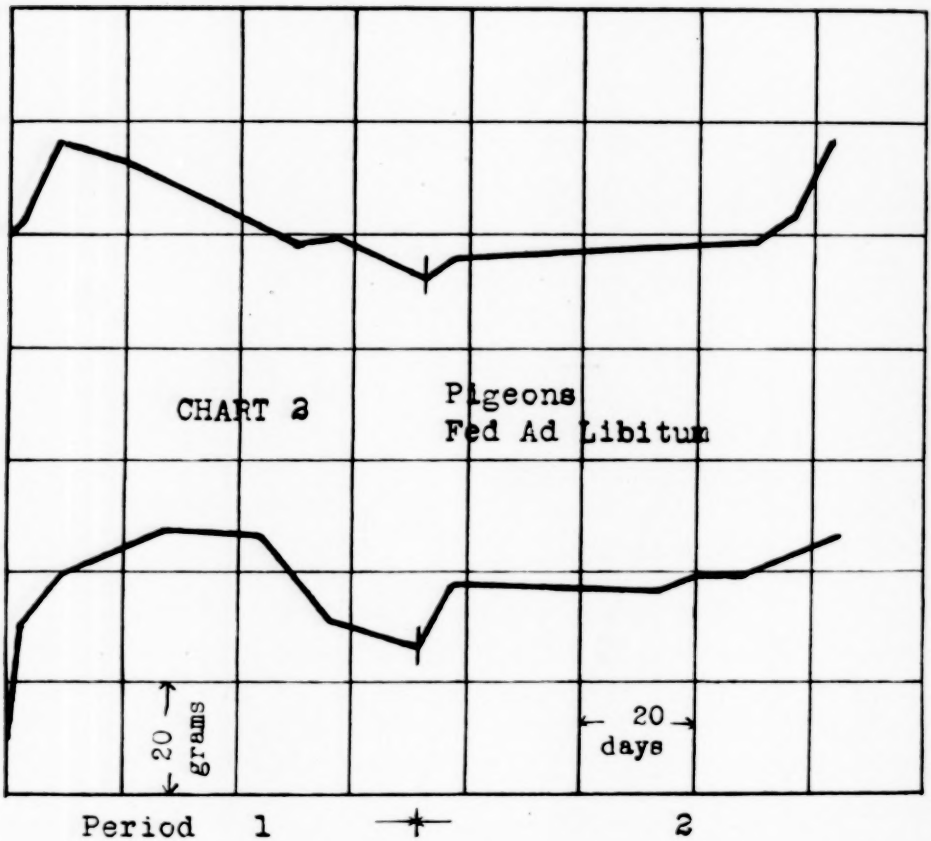

CHART 2. Shows the average weight curves for pigeons that were fed *ad libitum*. Period 1 unmilled rice heated for 2 hrs. at 120°C. in the air oven and Period 2 on the rice that was heated in the autoclave 1 hr. at 120°C. There was a gradual loss in weight on the dry heated rice, and only slight symptoms of polyneuritis. Following this, the pigeons gained slightly on the 1 hr. autoclaved rice, just as they did in Chart 1.

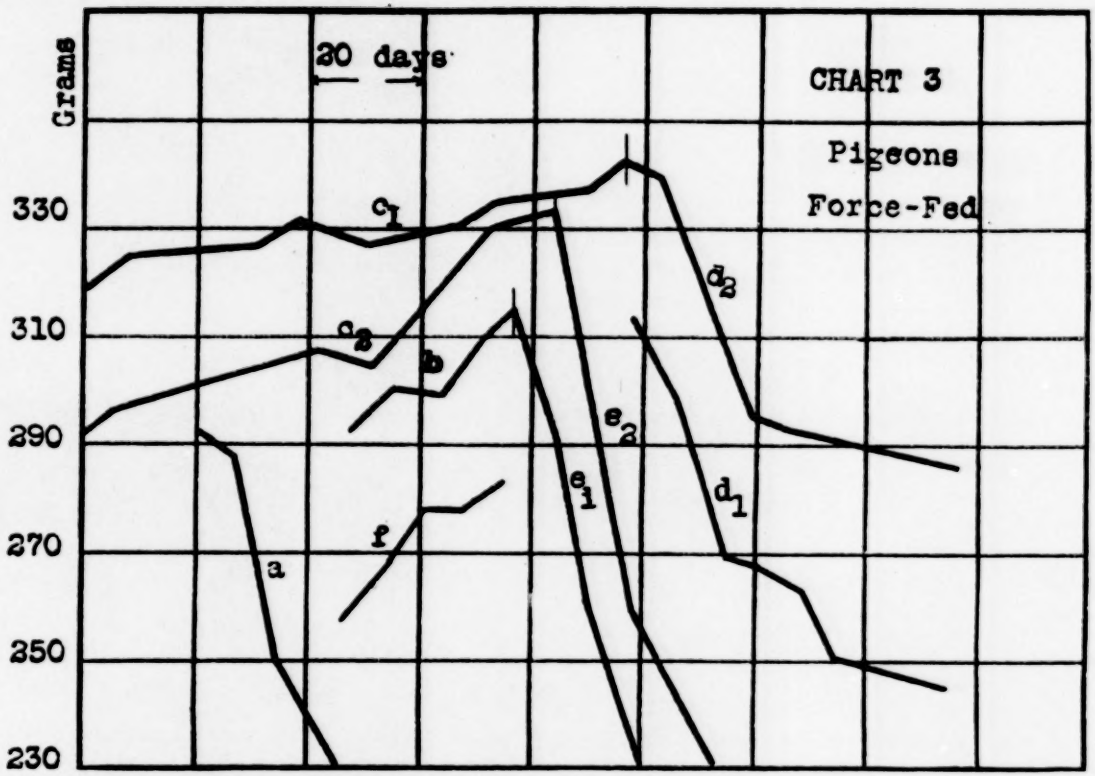

CHART 3. Pigeons were first given *ad libitum* unheated and 1 hr. autoclaved rice (Curves b, c_1, and c_2) respectively. After a time they were force-fed, in some cases on the 2 hrs. autoclaved rice and in others on the 6 hrs. heated rice (Curves d_1, d_2, e_1, and e_2, respectively). In Curve a, they were force-fed the milled rice as a control.

When they consumed the unheated rice they gained, and lost rapidly when force-fed on the 6 hrs. heated rice. Likewise, they gained on the 1 hr. autoclaved rice and lost on the 2 and 6 hrs. heated rice. The pigeons regurgitated some of their food when force-fed, but retained more of it than when they were fed *ad libitum* in Chart 1. That this loss in weight was not due to the mechanical handling of the pigeons in force-feeding them is evidenced from Curve f where they were force-fed on unheated unmilled rice and gained.

The curves show that even though the pigeons consumed more of the 2 and 6 hrs. autoclaved unmilled rice than when fed *ad libitum*, the extra amount had no value.

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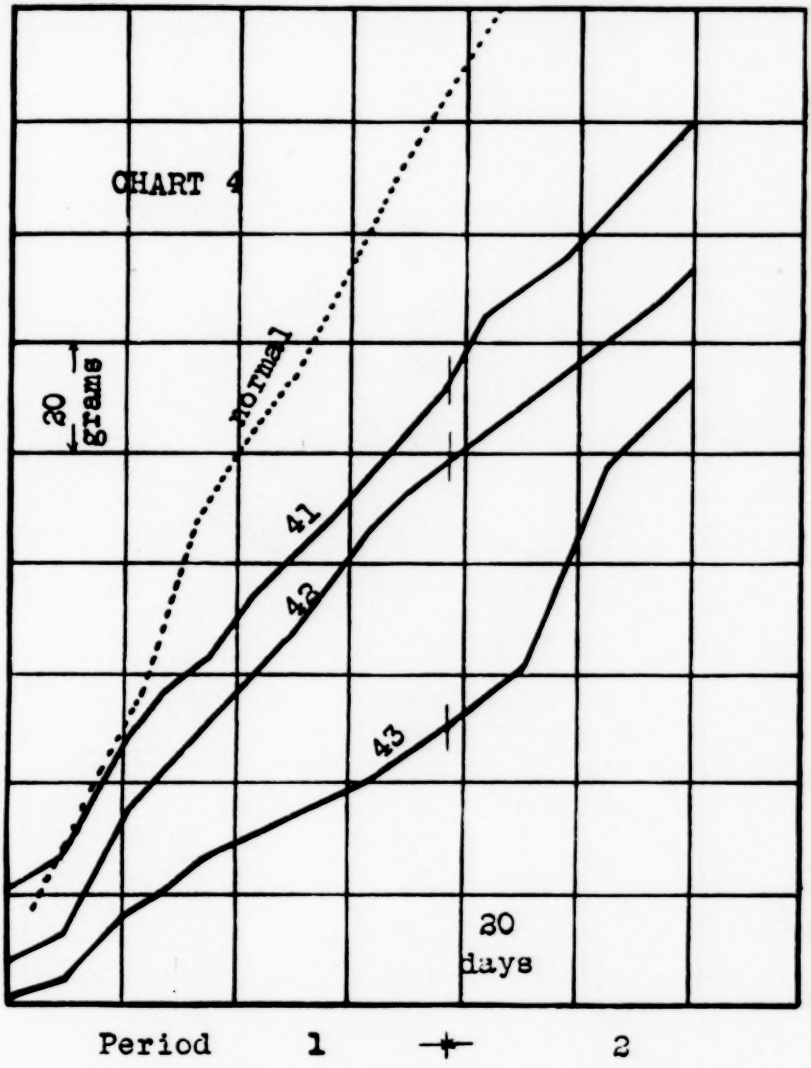


CHART 4. The rats were fed the basal diet of unheated unmilled rice, supplemented with lactalbumin, salt mixture, butter fat, and lard. The protein plane was 10 per cent. In Period 1 the rice formed 64 per cent of the ration and in Period 2, 89.7 per cent.

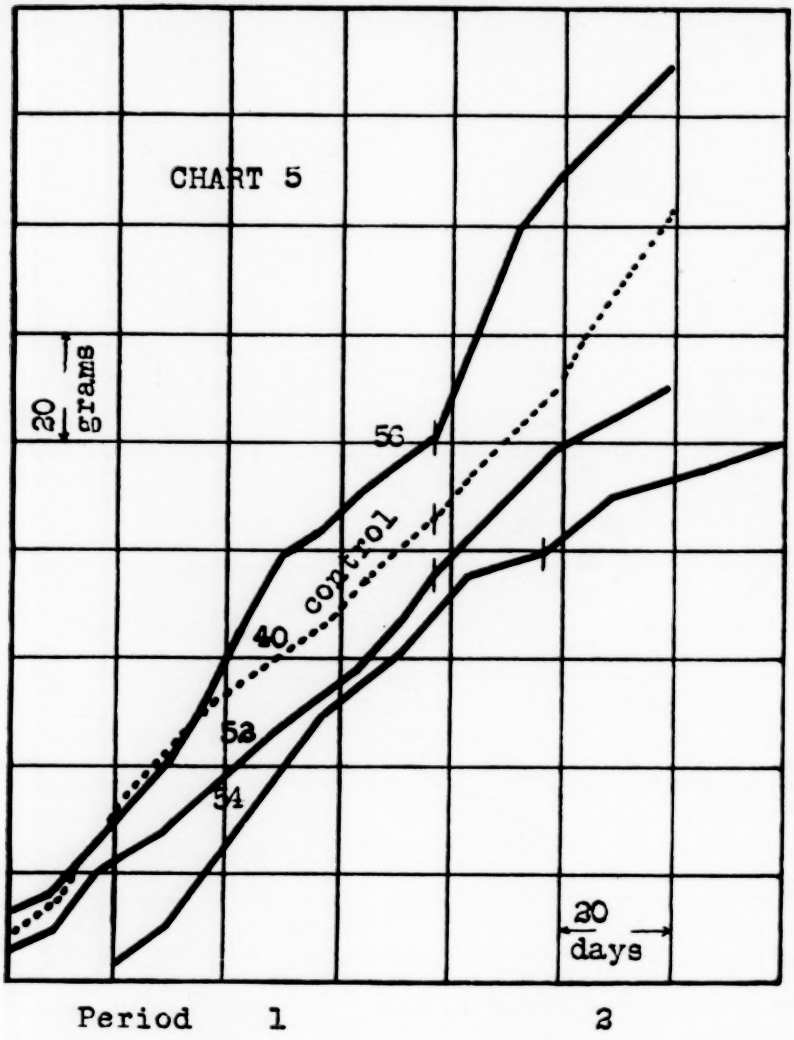


CHART 5. The rats were fed the basal diet of unmilled rice heated 2 hrs. in the air oven at 120°C. Otherwise the ration was the same as for Chart 4. The control, Curve 40, is the average for the curves for the unheated rice (Chart 4).

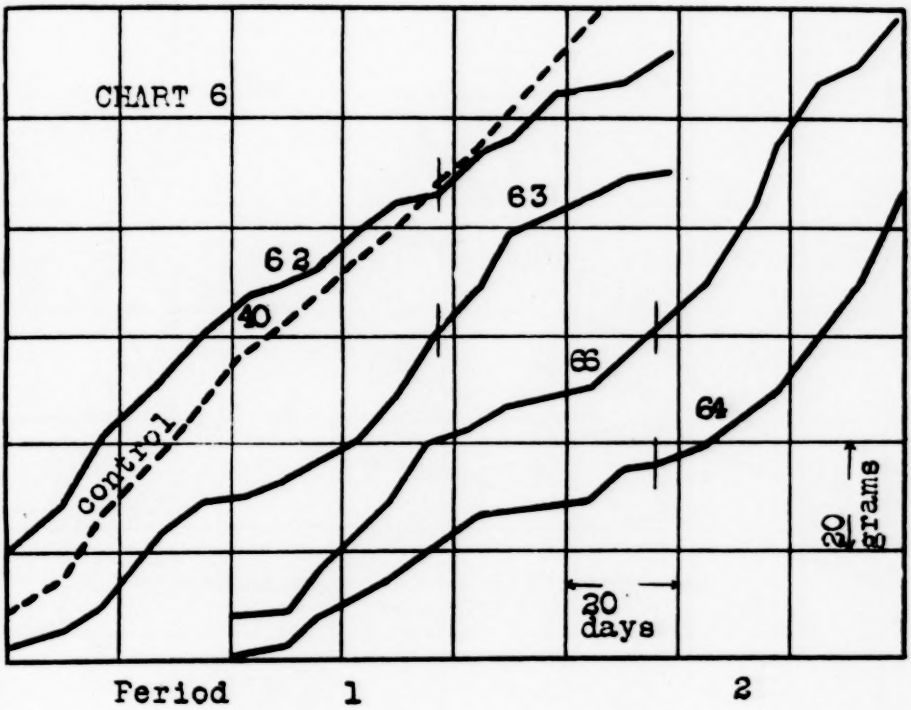


CHART 6. Same as Chart 5, except the basal rice diet was heated 2 hrs. in the autoclave at 120° and 15 pounds pressure.

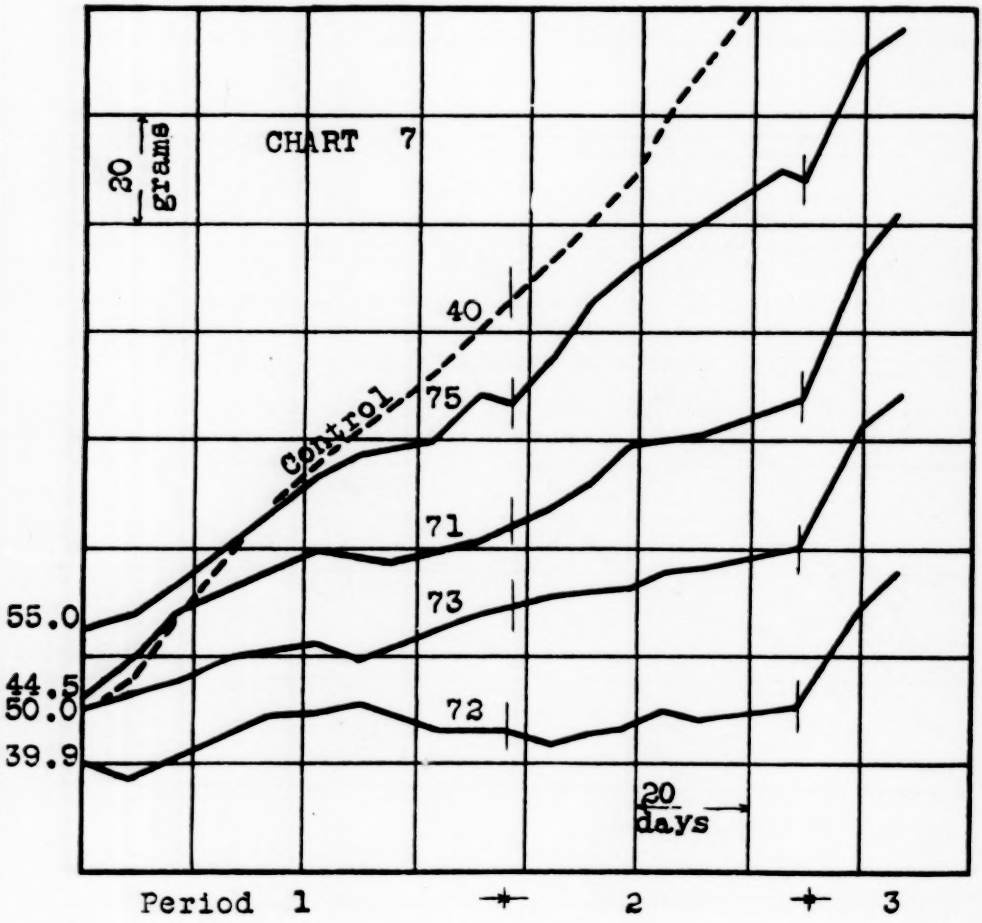


CHART 7. Same as Chart 6, except the rice was heated for 6 hrs. in the autoclave at 120° and 15 pounds pressure. In Period 3, the rats were fed the same ration as in Chart 4.

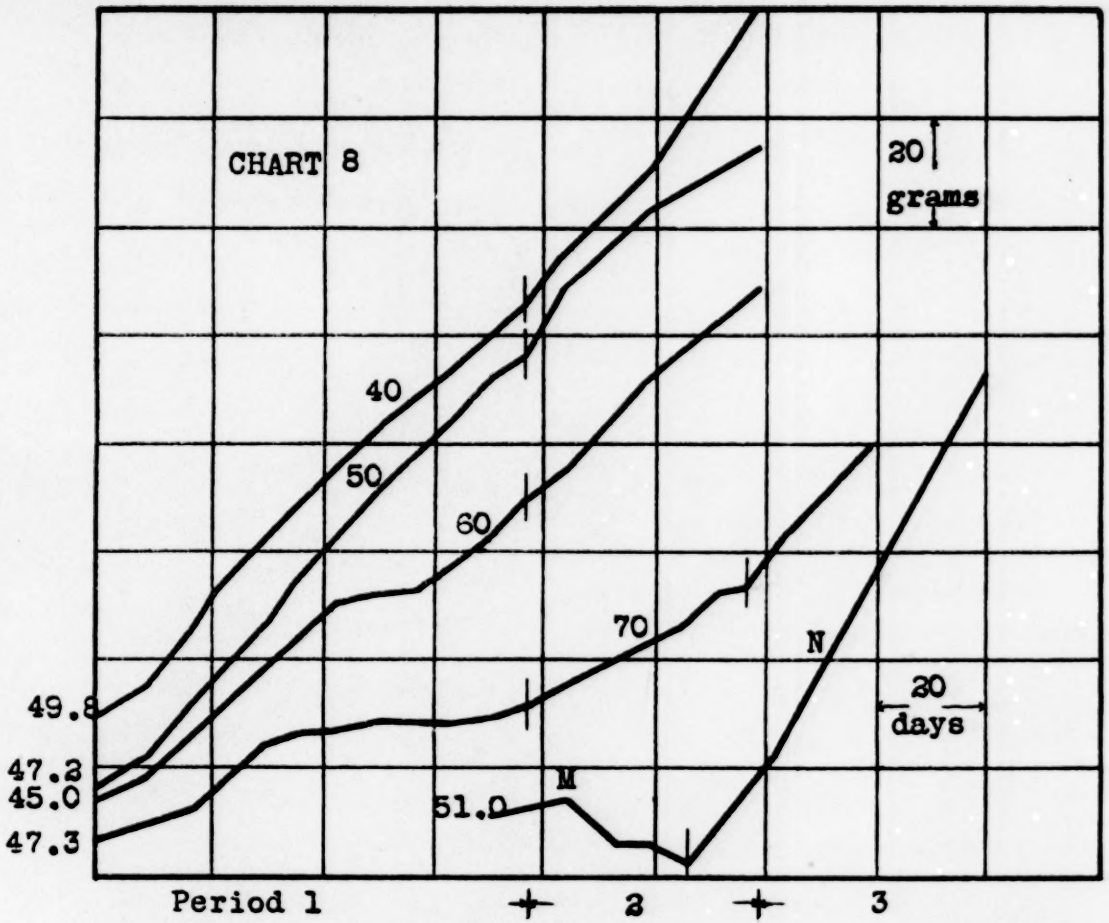
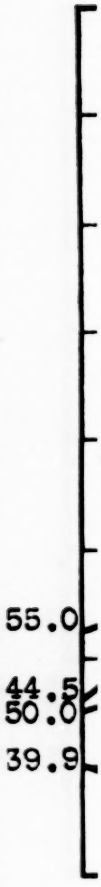


CHART 8. The average curves show that the growth-promoting vitamine was not altered by heating at 120° for 2 hrs. in the oven. Thus, Curve 50 ran parallel with that for the unheated rice (Curve 40). In the case of the 2 hrs. autoclaved rice, heated at 120° and 15 pounds pressure, Curve 60 ran practically parallel with the other two, except for a short period. The curve for the 6 hrs. heated rice (Chart 70) shows some gain. In all four groups, there was a definite gain.

If the growth-promoting water-soluble vitamine had been destroyed by heat, the resultant effect in these four curves would have been a decline in weight the same as for the first part of the control, Curve *MN* where rats were first (*M*) fed on a ration composed of butter fat, lard, starch, lactalbumin, and salt mixture without any of the so called water soluble B, and later (*N*) given this vitamine.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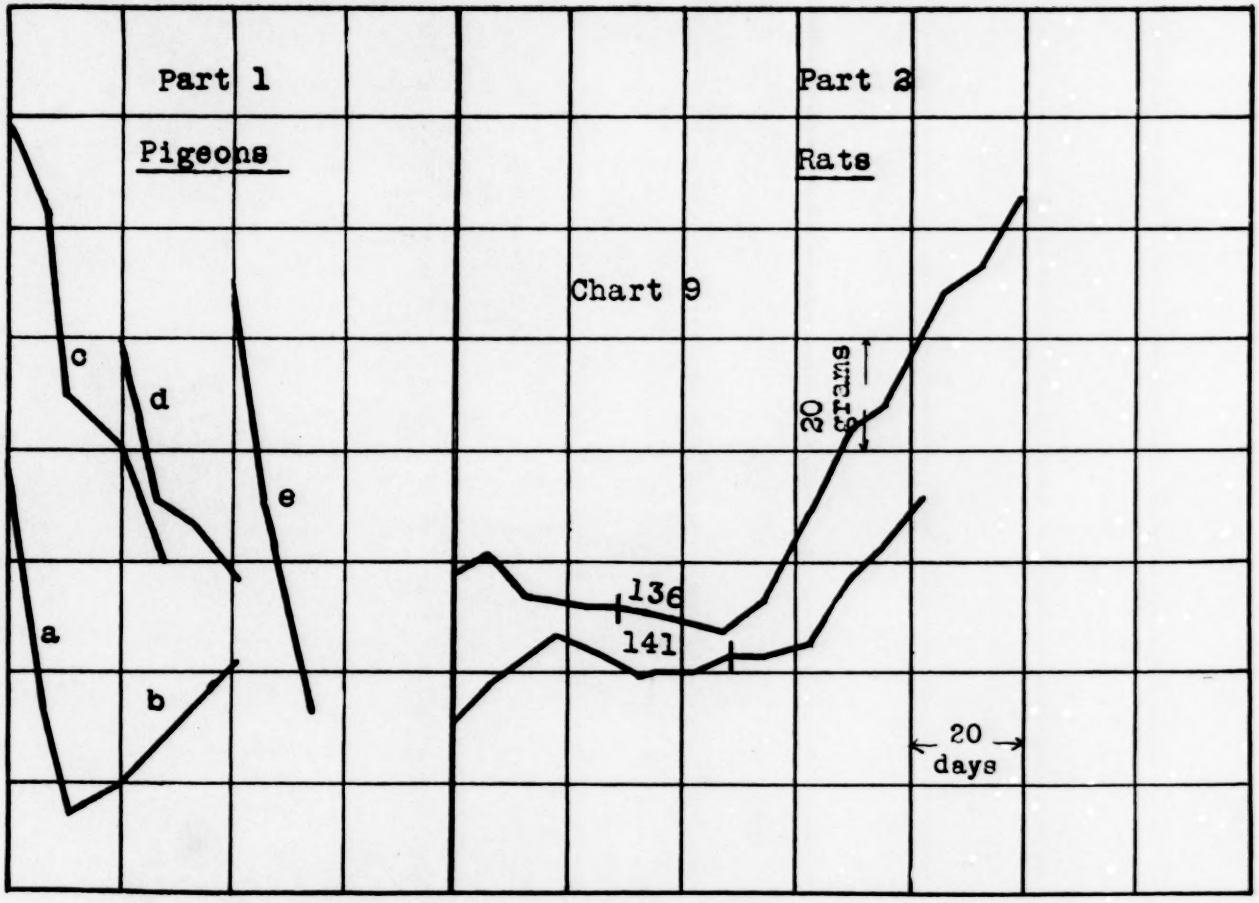
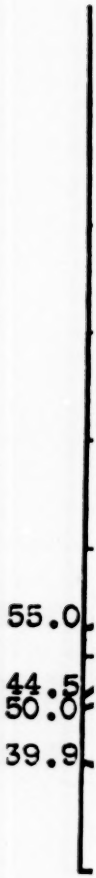


CHART 9. In Part 1, the pigeons were all force-fed milled rice. In the case of Curve a, they were not treated until there were definite signs of typical polyneuritis. Then the pigeons were treated with unheated vitamine extract. In Curves c, d, and e, the pigeons were treated from the beginning every other day with equivalent amounts of the extracts that had been heated 2 hrs. at 120° in the air oven, 2 hrs. at 120° in the autoclave, and 6 hrs. at 120° in the autoclave, respectively. The evidence is quite conclusive that heating under these conditions destroyed the vitamine. In fact, the vitamine in the extract was undoubtedly broken down more quickly than in the rice.

In Part 2, the same vitamine extract after heating 6 hrs. in the autoclave at 120° was incorporated in a ration that was deficient in the water-soluble growth-promoting factor. The fore period shows that the rats were not growing while the test period indicates that they began to grow after adding the extract indicating that this vitamine was not totally destroyed by the heating.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WATER-SOLUBLE VITAMINES.

II. THE RELATION OF THE ANTINEURITIC AND WATER-SOLUBLE B VITAMINES TO THE YEAST GROWTH-PROMOTING STIMULUS.*

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(From the Biological Research Laboratory of Parke, Davis and Company, Detroit.)

(Received for publication, May 22, 1920.)

With the idea of making a further study (1) as to whether the antineuritic and the water-soluble B vitamines were different, we endeavored to apply one of the two yeast quantitative methods that have been proposed. We selected the Williams (2) micro method which the author claims measures qualitatively and quantitatively the presence of the antineuritic vitamine. Bachmann's fermentation method (3) has been used by Eddy (4).

The plan of approaching this phase of the problem was similar in some respects to that which we used formerly (1); that is, a study of the effect of heat on the water-soluble vitamines. Comparative trials were made to determine whether or not the extracts that caused increased growth of the yeast would cure polyneuritis in pigeons, or excite growth in young rats that were suffering from a lack of the water-soluble B vitamine.

Briefly, this micro method[1] was as follows: (*a*) Preparation of synthetic media—saccharose, 20 gm.; $(NH_4)_2SO_4$, 3 gm.; KH_2PO_4, 2 gm.; asparagine, 3 gm.; $CaCl_2$, 0.25 gm.; and $MgSO_4$, 0.25 gm., all made up to 1 liter with distilled water. The reagents were purified. The media was sterilized at 10 pounds pressure for 10 minutes and kept in the refrigerator. (*b*) Procedure: A suspension was made of yeast cells in about 30 cc. of sterile distilled water. Duplicate test solutions were prepared quantitatively by taking 25 cc. of the synthetic media and 1 to 5 cc. (depending upon the concentrations) of a definite solution of the unknown extract. This was

* Proceedings of the American Chemical Society, St. Louis, April, 1920.

[1] The authors desire to express their appreciation to Professor F. C. Koch, of the University of Chicago, for the privilege of permitting us to become familiar with the technique of the details of this method.

diluted to 30 cc. (using sterile water if needed). The mixture was sterilized and 1 cc. of the yeast suspension added. After mixing, thirty-six drops were made with a fine pen point on a cover slip that had been coated with a very thin film of purified vaseline. This slip was inverted and sealed tightly on a hanging drop slide. Each drop was examined for single yeast cells, the observations were recorded, and the time was noted. The slide was then placed in the incubator at 30°C. and the readings were made again in 18 hours. In case the number of cells in each drop at the end of this period exceeded 75, the determination was repeated using less of the unknown. Similar tests were run on the synthetic media as a control. After correcting for this blank determination, the rate of growth in terms of yeast cells was calculated per gm. of the original unknown material.

TABLE I.

Preliminary Tests with the Williams Micro Yeast Method.

Concentration of solution of vitamine extract No. 2650.	Amount added to media.	Average reading of yeast cells.*	Yeast cells per gm. of original substance.	Average of duplicate cells per gm. of substance.
per cent	*cc*			
A. 0.02 made from a 5 per cent solution by dilution.	1.0	7.8	39,000	
	1.0	8.1	40,500	39,750
B. 0.02 made from a 5 per cent solution by dilution.	3.0	23.8	39,500	
	3.0	23.6	39,000	39,250
C. 0.02 made directly.	3.0	23.6	39,000	
	3.0	24.0	40,000	39,500
D. 0.2 made from a 5 per cent solution by dilution.	3.0	82.2	38,600	
	3.0	82.4	38,700	38,650
Average				39,287

* Corrected for control reading on reagents.

Table I gives an idea as to the accuracy of the Williams method. It will be seen, in varying the concentration of the solution, or in taking different quantities of the same solutions, that not only the duplicates in any one series but the corresponding final average values in the different series agreed remarkably closely for such a biological method, showing that this method can be used with definiteness for measuring the rate of growth of yeast.

DISCUSSION.

The Yeast Growth-Promoting Factor Is Not the Antineuritic Vitamine.—In making these tests, natural or unmilled rice was heated as follows: 1 hour in the autoclave at 120° and 15 pounds pressure; 2 hours in the autoclave at 120°; and 6 hours in the autoclave at 120° and 15 pounds pressure. These rices, together with some of the unheated rice, were finely ground, then extracted with hot 95 per cent alcohol. These extracts were concentrated *in vacuo*, taken up with hot water and salt, then filtered, and made up to a definite volume. The tests were then made upon

TABLE II.

Effect of Heat on Yeast Growth-Promoting Vitamine.

Extracts of natural rice.	Average reading of each determination.*			Average yeast cells per cc.	Yeast cells per gm. of original rice.
	1 cc.	3 cc.	4 cc.		
Unheated rice.	12.7	38.0			
	12.3	38.7		12.6	63
1 hr. in autoclave at 120°C.	12.9				
	13.1			13.0	65
2 hrs. " " " 120°C.	12.8	37.7			
	12.9	37.1		12.6	63
6 " " " " 120°C.	13.1		49.6		
	13.1		49.0	12.7	64

* Corrected for control reading on reagents.

these solutions, blank determinations being carried out with the same dilution of salt and synthetic media. Corrections were applied in making the final calculations. No data are included with respect to comparing heated extracts that originally contained the antineuritic vitamine. We found that this extracted vitamine was apparently more unstable to heat than when in the natural food.

The data are presented in Table II. They indicate very clearly that the factor which stimulated growth in the yeast was not altered in the least by the process of heating. This obser-

vation was contrary to what we had expected, since Williams (2) designated the yeast growth stimulus as the antiberi-beri vitamine. From previous findings (1) we have already concluded that the extensive heating of the rice (2 and 6 hours in the autoclave at 120° and 15 pounds pressure) altered in a very marked way the potency of antineuritic vitamine. Therefore, we naturally expected the activity of the extracts, in terms of yeast cells, to decrease with the higher degree of heating.

TABLE III.

Potency of Extracts from Natural Rice on Polyneuritic Pigeons.

Extract of natural unmilled rice.	Pigeon No.	Equivalent yeast cells given in extract.	Yeast cells per gm. of body weight.	Response to treatment.
Unheated.	510	10,269	41	Cure.
	803	7,812	31	"
Average.			36	"
2 hrs. in autoclave	805	9,750	38	No improvement, died.
at 120°C.	793	9,750	38	" " "
Average.			38	" " "
6 hrs. in autoclave	735	10,400	42	No improvement, died.
at 120°C.	781	10,400	33	" " "
Average.			38	" " "

In order to prove this point more definitely, the antineuritic property of the extracts of these rices was tested by treating polyneuritic pigeons with equivalent quantities of each extract in terms of the number of cell units per gm. of body weight. These data are presented in Table III.[2]

There is, of course, a possibility that in the heating of the rice some toxic substances were formed which were removed in the extraction along with the vitamine and in turn prevented the antineuritic vitamine from acting on pigeons. If so, one would expect the yeast growth-promoting vitamine to be affected in

[2] In making these tests the pigeons selected showed the usual definite signs of typical advanced polyneuritis. The "cure" cases remained positive for 7 to 10 days which is sufficient for a definite test of the potency of an extract.

the same manner, but it was not. On the other hand, with the yeast cell stimulus, we have found that some extracts contain toxic factors although the antineuritic and the water-soluble B vitamines were shown to be present by biological tests. As a result, the readings were very misleading as shown in Table IV.

The Yeast Growth-Promoting Factor Apparently Does Not Stimulate Growth in Young Rats.—In order to obtain an idea as to the amount of the yeast stimulus that normal rats require, the value

TABLE IV.

Toxicity in Relation to the Yeast Growth-Promoting Stimulus.

No. of extract.	No. of yeast cells in diluted solution.	
	1 cc.	3 cc.
30–40	12.7–12.8	7.8–8.7
	12.75	8.25
30–50	10.9–11.0	7.3–8.9
	10.95	8.1
1,339 (2 hrs. in autoclave at 120°C.)	9.5–13.5	11.9
	11.5	
1,339 (6 hrs. in autoclave at 120°.)	13.2–13.9	11.7–11.9
	13.55	11.8

was calculated from previous prophylactic feeding experiments where rats were fed as their basal diet natural rice (1). The data are given in Table V.

In making the tests to determine whether or not the rats would be stimulated to grow on a definite number of yeast cell units, a water-soluble vitamine preparation was activated with fuller's earth by the Seidell method (5). The filtrate was found to contain 2,537 yeast cell units or 6.4 per cent of the total yeast factor. Failure to cure pigeons showed that its antineuritic vitamine content was extremely low.

TABLE V.

Calculated Yeast Cell Units Consumed by Normal Rats.

Substance.	Group of rats.	Yeast cell units per day per gm.	Remarks.
Natural rice, unheated.	40	3.0	Gained in weight.
" " 2 hrs. dry heat at 120°.	50	3.3	" " "
Natural rice, 2 hrs. autoclaved at 120°.	60	3.3	" " "
Natural rice, 6 hrs. autoclaved at 120°.	70	2.7	Moderate gain in weight.

Rats that had been brought down to a low nutritive plane, due to a lack of the so called water-soluble B, were treated with 1 to 1.5 cc. of this filtrate. As the rats were weighed daily, it was found (Table VI) that Rats 834 and 835 both lost in weight during the 15 days treatment. Further, in the case of Rat 735, its loss in weight was so marked that we were forced to give it a treatment of the original vitamine extract whereupon it immediately began to gain.

Following the first treatment of Rats 834 and 835 with an extract from the 6 hours autoclaved 120° natural rice, they stopped

TABLE VI.

Relation of Yeast Cell Units to Growth of Rats.

Substance.	Rat No.	Yeast cell units per day per gm.	Remarks.
Extract 7,000.5b-Filtrate fullers' earth.	834	7.0	Weighed 47 gm. Lost 4.2 gm. in 15 days.
Extract 6 hrs., autoclaved 120° natural rice.	834	7.5	Gained 3.0 gm. in 5 days.
Extract 7,000.5b-Filtrate fullers' earth.	835	7.8	Weighed 35.5 gm. Lost 4.0 gm. in 15 days.
Extract 6 hrs., autoclaved 120° natural rice.	835	6.9	Gained 10 gm. in 5 days.
Extract 7,000.5b-Filtrate fullers' earth.	735	15.3	Weighed 49.5 gm. Lost 6.4 gm. in 3 days.

losing and began to gain on doses that were equivalent in yeast cell units to what they were given of Extract 7,000.5b.

In Table VII, further data are presented with respect to the amount of extract, in terms of yeast cell units, that were required to make a rat grow. These two rats, Nos. 673 and 675, were so far down from a lack of the water-soluble B that they had reached that condition which is described by some as polyneuritis.

It is thus seen that the rats in the curative or corrective tests (Tables VI and VII) were given 2 to 2.5 times as much of the

TABLE VII.

Relation of Yeast Cell Units to Growth of Rats.

Substance.	Rat No.	Yeast cell units per gm.	Remarks.
Extract 2 hrs. autoclaved 120° natural rice.	673	14*	Weighed 7.2 gm. Lost 9.2 gm. in 5 days.
Extract unheated natural rice.	673	14	Gained 25 gm. in 19 days, following one treatment only.
Extract 2 hrs. autoclaved 120° natural rice.	675	14*	Weighed 99.7 gm. Gained 11 gm. in 5 days.
Extract unheated natural rice.	675	14	Gained 20 gm. in 19 days, following one treatment only.

* Given every 2nd day.

yeast cell units in Extract 7,000.5b as those on the prophylactic tests (Table V) yet they did not respond and grow. This suggests that either the yeast cell stimulus was not the same as the water-soluble B, or that the rats in this condition required more of it. When the dose was increased to almost five times, Rat 735 refused to grow. This led us to infer that there was some factor involved in the growth of the rat other than the yeast cell units, or at least to conclude that the yeast cell stimulus was not in itself able to retard the loss in weight and excite growth, measured by increase in weight of rats. As a further proof of this, the data in Table VII tend to show that, with the amount

and kind of extract used, the water-soluble B vitamine had a specific action on the growth of the rat that the yeast stimulus lacked.

This statement is not intended to convey the idea that the stimulus or vitamine which certain varieties of yeast seem to utilize is not needed by the rat or the pigeon for some other definite purpose or purposes in their physiological economy. Our data do not lend themselves to an interpretation of this point.

CONCLUSIONS.

1. The rate of growth of the yeast which we employ is strikingly accelerated by the addition to the synthetic media of very small amounts of preparations, which we have shown by biological tests to contain the antineuritic and the water-soluble B vitamines, provided certain toxic substances are absent.

2. The yeast growth-promoting factor does not appear to be the same as the antineuritic or antiberi-beri vitamine (pigeons).

3. This yeast stimulus is also possibly different from the water-soluble B growth-promoting vitamine (rats).

4. Whether pigeons or rats require this yeast growth-promoting factor for normal development has not, as yet, been definitely proved.

5. Since the amount of yeast growth stimulus, expressed in terms of yeast cell units per gram of substance, does not appear necessarily to vary directly (in terms of potency) with the antineuritic and water-soluble B vitamines, this yeast method should not be employed quantitatively with too much definiteness until further study is made.

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STUDIES IN THE VITAMINE CONTENT.

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In 1917 the results of experiments conducted at the New York Hospital with vitamine B dosage in cases of infant malnutrition demonstrated the necessity for some method of controlling this dosage. At that time the senior author reported some experiments to this end, using the Folin-Macallum uric acid reagent for the purpose.[1] This work was interrupted by the war and on resuming investigation in the fall of 1919 attention was directed to two publications bearing upon this subject and suggesting the utilization of the vitamine requirement of yeast as the measuring medium.[2]

In the Fall of 1919 we began a systematic investigation of these two methods to determine their practicability for the measurement of the B vitamine. Our earlier studies were with the Bachmann technique followed by a similar study of Williams'. Our observations led finally to the evolution of a new technique which utilizes features of both these authors' methods but we believe eliminates some of the features that made their tests difficult of quantitative control. The various steps have been reported from time to time[3] but are here collated in full.

The Practicability of the Bachmann Test.

The methods adopted were practically those suggested by Bachmann. Fermentation tubes were filled with Nägeli solu-

[1] Eddy, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1916–17, xiv, 164.

[2] Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235. Williams, R. J., *J. Biol. Chem.*, xxxviii, 465.

[3] Eddy, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 52, and two other articles in press.

tion (100 cc. of distilled water, 10 gm. of dextrose, 1 gm. of ammonium nitrate, 0.05 gm. of calcium phosphate, 0.5 gm. of potassium acid phosphate, 0.25 gm. of magnesium sulfate) and sterilized in the Arnold sterilizer. The vitamine extracts were prepared from navy bean by the method described by McCollum and Simmonds.[4] This method yielded the vitamine in alcohol extract, and this extract was either evaporated down on dextrin and a water solution of the activated dextrin used or was simply evaporated to dryness and a water solution of the residue used. The concentrations were controlled by varying the amount of

TABLE I.

Tube No.	Tube contents.	Per cent of gas formation by days.								
		1	2	3	4	5	7	8	9	14
1. Control.	Nägeli solution plus yeast (Control 1).	0	0	0	0	0	0	0	0	0
2. "	Nägeli solution plus yeast plus 1 cc. dextrin solution (Arnold).	0	0	0	0	0	0	0	0	0
3. "	Nägeli solution plus yeast plus 1 cc. dextrin (Arnold and autoclave).	0	0	0	0	0	0	0	0	0
4. Test.	Nägeli solution plus yeast plus 1 cc. dextrin-vitamine solution (Arnold).	0	90	87	63	18	Discontinued.			
5. "	Nägeli solution plus yeast plus 1 cc. dextrin (Arnold and autoclave).	0	93	85	56	56	40	37	37	33

water. All such extracts were sterilized in the Arnold sterilizer and the sterile extract was introduced in appropriate amounts into the fermentation tubes. The tubes were finally inoculated by a loopful of a yeast suspension made from a pure culture of Fleischmann yeast. In certain cases unextracted material was used but in all cases the material was sterilized before testing. The first results are shown in Table I. In the experiments a watery solution of the activated dextrin obtained from navy bean was used. The solutions in Tubes 2 and 3 consisted of water solutions of unactivated dextrin of the same concentration as the

[4] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, xxxiii, 55.

dextrin in Tubes 4 and 5. Two methods of sterilization were used, one (Arnold) being a submission of the extract to two 30 minute periods in the Arnold sterilizer at 100°C., 24 hours elapsing between the two periods, while the other (Arnold and autoclave) added a third 30 minute period in the autoclave at 15 pounds pressure and approximately 120°C.

These first results demonstrated Bachmann's point that gas formation fails to result in the absence of an extract containing the B vitamine, if we assume that the substance in the extracts of Tubes 4 and 5 responsible for the effects is the vitamine. The reduction in gas on successive days is due to the absorption of the

TABLE II.

Comparison of Concentrations by the Bachmann Method.

Concentrations.	Arnold-sterilized solutions.												Arnold- plus autoclave-sterilized solutions.											
	Series I.					Series II				Series III.				Series I.					Series II.				Series III.			
Days..	1	2	3	4	5	1	2	3	4-5	1	2	3	4-5	1	2	3	4	5	1	2	3	4-5	1	2	3	4
cc.																										
1	0	90	87	63	18	0	99	100	85	0	68	100	82	0	95	85	58	56	0	99	100	65	0	68	100	80
0.5	0	95	91	85	68	0	95	100	99	0	53	95	95	0	26	51	58	25	0	60	100	84	0	23	100	96
0.2	0	16	63	29	11	0	2	35	29	0	1	78	99	0	50	70	35	72	0	0	18	30	0	0	75	67
0.1	0	0	0	0	0	0	0	0	1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0

CO_2 back into the medium on standing, probably a siphoning action which begins as soon as saturation is complete. This feature is one of the hindrances to accurate quantitative comparison by this method but if this had been the only objection it could have been easily obviated.

The results in Table II indicate that below the optimum concentration the gas formation varies with the concentration and was the first indication that in comparing solutions it is first necessary to determine the dilution necessary to produce optimum results since amounts above that do not appreciably affect the strength of the reaction. In the above tests watery solutions of activated dextrin with the navy bean as source were used. The variations in the different series were probably due to the amount of yeast used in

the inoculations and pointed out the necessity of standardization in this particular for truly quantitative comparisons. The autoclave seems to reduce the activity slightly when the concentration is under the optimum but the results in this respect are extremely inconclusive from the evidence given above. The tests were ended when the maximum gas production was reached.

A Cream of Wheat extract was made in the same manner and with the same amount of wheat as was taken of navy bean in the preceding tests. The results in Table III are interesting in showing that Cream of Wheat while largely endosperm responds markedly to the test. The protein-free milk was not from Walker-Gordon material; the amount used was comparable, however, to 1 cc. of whole milk. The variability of the vitamine content in milks of

TABLE III.

Application of the Method to the Qualitative Analysis for Vitamine.

Substances tested.	Per cent gas formation by days						
	1	2	3	4	5	6	7
1 cc. vitamine extract of Cream of Wheat.....	0	60	100	83	33	28	
$\frac{3}{5}$ " protein-free milk.....	0	20	40	53			100
1 " Walker-Gordon whole milk.....	0	100	100	100	100	60	
1 " " " " " (duplicate)...	0	68	100	100	66		
0.5 " " " " "	0	52	100	68	50		
0.3 " " " " "	0	25	60	50	32		

different sources is well borne out by this experiment. Arnold sterilization alone was used on all the above products. The results in Table IV leave no doubt that cow's milk is richer in vitamine than human milk in the concentrations used; that is, without dilution or concentrating. When, however, we attempt to compare the results of the different tests the variability in the human milks is disconcerting. In Series I of Table IV the strength is ACDBEF. In Series II with the same concentration it is ADECBF. When the amounts are reduced to 0.5 cc. as in Series III the results are (BD)EFCA. Such variations indicate that when vitamine contents are of nearly the same magnitude the test is unreliable in the form in which we have used it.

From these and other tests that need not be recorded here, we feel that the Bachmann technique is in need of considerable modi-

fication if it is to be used for quantitative comparison of vitamine content. Furthermore, since the activity of an enzyme is so easily varied by hydrogen ion concentration and by other factors, we abandoned this method at this point to experiment with the Williams technique.

TABLE IV.

Comparison of Walker-Gordon Whole Milk (Cow) and Six Samples of Human Milk from Six Different Sources; Bachmann Method.

Substances tested.	Per cent of gas formation by days.														
	Series I.					Series II.					Series III. (0.5 cc.).				
	1	2	3	4	5-6	1	2	3	4	5-6	1	2	3	4	5-6
1 cc. Walker-Gordon cow milk	0	100	100	100	60	0	68	100	100	86	0	52	100	68	50
1 cc. breast milk A	0	12	63	80	100	0	0	60	100	68	0	0	100	10	30
1 " " " B	0	21	59	70	70	0	31	70	72	62	0	0	24	65	90
1 " " " C	0	17	47	68	82	0	8	82	60	65	0	3	32	50	48
1 " " " D	0	31	72	72	72	0	65	90	80	70	0	0	28	78	90
1 " " " E	0	1	23	56	65	0	5	70	90	65	0	0	20	68	64
1 " " " F	0	0	20	35	59	0	3	78	72	45	0	0	48	68	50

Experiments with the Williams Technique.[5]

By substituting for gas formation the actual increase in yeast cells as the unit of measurement, this test eliminates the variability in enzyme activity as a controllable factor. In practice we found it was an exceedingly difficult matter to dip out with a pen, as directed by the author, drops containing uninjured single cells. Secondly, it was not easy to be sure of our count under the microscope and, finally, incubation resulted in evaporation and made it difficult to control accurately the quantities of extract measured for the test. For all these reasons we sought a simpler method, more easily controllable but retaining the cell count method for comparisons.

[5] At the time this article was submitted, the gravimetric method (Williams, R. J., *J. Biol. Chem.*, 1920, xlii, 259) had not appeared.

A New Technique for the Measurement of Vitamine Content.

Fig. 1 shows the tools used, being essentially those of the opsonin technique. The first step in the preparation is the manufacture of a pair of capillary pipettes. These are made by drawing out in the flame a piece of 5 mm. soft glass tubing. At the center of the capillary portion a mark is made and with a drop of

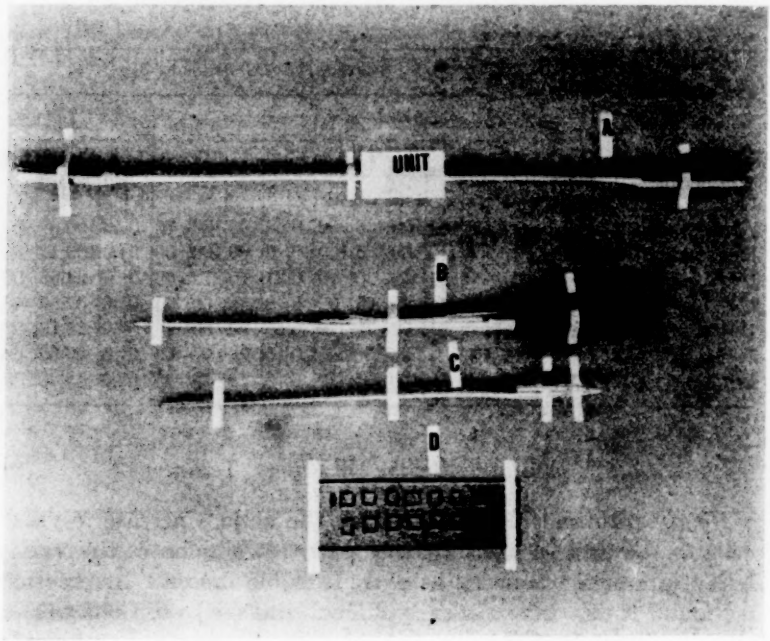

Fig. 1.

mercury two units are marked off on each side of this center. Our unit was chosen arbitrarily and consisted of a drop of mercury weighing 0.0108 gm. and occupying a volume of approximately 0.0007 cc. The tubes, after calibrating, were cut apart at the center. Each pipette is then constricted in the flame at a point near the large end to permit of a flame seal here later. The large end is then plugged with cotton and the tube sterilized. By fitting a rubber bulb to the large end it is ready for use.

Method.

The materials used in the test are the capillary pipette described above, a dilute suspension of yeast cells in Nägeli solution, and a sterile solution of the vitamine extract to be tested. Miss Stevenson investigated some thirty strains of yeast to determine their relative suitability for the test and found that the one obtainable from a Fleischmann yeast cake was as good as any for the purpose. Since this yeast is readily available at all times we adopted it for our purpose. A pure culture of this yeast is maintained on an agar slant. 48 hours before beginning the test a transplant is made to fresh agar and at the end of the 48 hours as small a portion as can be taken up on the tip of a needle is transferred to 10 cc. of Nägeli solution. The tube is then shaken for 2 to 3 hours in a mechanical shaker. This process does not give absolutely uniform suspensions and occasional clumps are found. Slow centrifuging will remove most of the clumps, however, and this method has been found satisfactory for results if controlled by a sufficient number of tests. For the perfection of the test it is eminently desirable to find a method of making a uniform yeast suspension and we are still experimenting with this difficulty. The uniformity of the suspension is tested before use by drawing up with the pipette five to ten units of the suspension and blowing them out on a glass slide where they are fixed, stained, and the cells counted. If the test shows fair uniformity the suspension is used, otherwise it is shaken further and the test repeated.

As soon as a fairly uniform suspension is obtained, the rest of the process is simple. First, prepare and sterilize as many pipettes as are needed for the test. Second, sterilize the vitamine extracts to be studied. When all are ready draw up into the pipette one unit of yeast suspension and one unit of the vitamine extract, mix by manipulating the bulb, seal the tip of the pipette in the flame, and make the flame seal at the constriction. The tube is now ready to incubate. The time of incubation varies with the strength of the solution to be tested but we have usually incubated the tube 20 hours at 35°C. At the end of that time the tips are broken at each end of the pipette, a bulb is placed on the large end, and the contents are blown out on a slide, fixed, and stained. The counting can then be done accurately at leisure. For a count-

ing slide we have etched 5 mm. squares on an ordinary microscope slide as shown in Fig. 1. This size holds the contents of a pipette, permits ease of operation when counting with the mechanical stage, and allows the contents of ten to twelve pipettes to be placed on one slide.

For control, another series of pipettes is prepared and filled by drawing up a unit of the yeast suspension without the unit of vitamine. These are incubated and counted in the same manner as the test pipettes. Obviously the greater the number of pipettes used the more accurately the results can be plotted. Tables V to XIV illustrate the excellencies and defects.

TABLE V.

Series.....	I	II	III	IV	V	VI	VII	VIII	IX	Size unit.
No. units yeast suspension.....	1	1	1	1	1	1	1	1	1	*cc.* 0.0007
No. units vitamine.....	2	2	2	2	2	2	1	1	2	0.0007
Period of incubation, *hrs.*.. ..	24	24	18	18	18	18	18	18	6	
Result (vitamine).	6,312	3,192	6,161	9,724	6,348	8,531	472	362	24	
" (control)...	5	28	14	123	46	97	58	60	2	

Evidence Bearing on the Specificity of the Test.

Table V demonstrates that our test was sensitive to small amounts of vitamine extract. Before going farther with the test, however, it was imperative that we demonstrate as far as possible the specificity of the reaction. To this end we applied the test to samples of the purified crystalline antineuritic vitamine prepared by Funk in 1912 and 1913 by his fractional precipitation methods, and kindly furnished by him for the pupose. The results of several tests are presented in Table VI. The marked activity of the 1913 (II) preparation was indicated, the inactivity of the 1912 sample, and the possible activity of the 1913 (I) portion. To settle this latter point new and more concentrated solutions were made of the 1912 and 1913 (I) preparations by dissolving 1 mg. in 0.3 cc. of water and testing a unit of each (Table VII).

TABLE VI.

Series	I	II	III	IV	V
1912 vitamine (1 unit (0.0007 cc.) of a solution contained 0.0124 gm. in 10 cc. of water)	2	10	11	3	7
1913 vitamine (I) (1 unit of solution contained 0.0145 gm. in 10 cc. of water)	59	31	41	4	10
1913 vitamine (II) (1 unit of solution contained 0.0065 gm. in 10 cc. of water)	404	68	322	27	35
Controls	113	15	56	4	4

TABLE VII.

Series	I	II	III	IV	V
1912 vitamine	5	6	87	89	10
1913 " (I)	206	182	190	149	230
Control			24		

The demonstrable impurity in these preparations was nicotinic acid and to determine whether that was the causative factor in the test results were obtained as given in Table VIII.

TABLE VIII.

Series	I	II	III	IV	V	VI	VII	VIII	IX	X	Average.
1 unit nicotinic acid solution made by dissolving 1 mg. in 0.3 cc. water	81	202	265	5	82	1	57	84	39	17	74.9
Controls (five pipettes)			127	94	64	3		151			84.0
Variations in ten units of the yeast suspension used	11	23	135	73	39	48	51	46	54	97	67.4

These results seem to indicate that the nicotinic acid is inactive since the average fails to exceed the controls and the variations are traceable to the variation in the yeast suspension used.

From these results it seems fair to conclude that the test works with small quantities of the Funk antineuritic vitamine as prepared by him in the purest form we have obtained to date and tends to strengthen the conviction that the antineuritic or B vitamine is the responsible causative agent in stimulating the growth of the yeast cells.

Our next step was to utilize the method reported for adsorption of the B vitamine by Seidell and Williams; *viz*, the selective adsorption of Lloyd's reagent. If this reagent adsorbs the B vitamine, a solution tested before and after treatment with the reagent should show marked difference in response. Our first tests in this direction were with a navy bean extract of the vitamine (Table IX).

TABLE IX.

Series	I	II	III	IV	V
Navy bean extract before treatment with Lloyd	1,927	2,333	672	633	11,237
The same extract after shaking with Lloyd	87	64	2	0	100
Controls	90	90	15	15	113

We next obtained from an orange, by sterile puncture, a portion of sterile juice. The sterility of the juice was determined by incubating on agar for several days. Portions of this sterile material were then tested before and after shaking with sterile Lloyd's reagent (Table X). It seems evident from these results that the

TABLE X.

Series	I	II	III	IV	V	VI
Orange juice before shaking	5,200	4,400	62,400	85,700	80,000	19,500
" " after "	169	176	109	240	301	133
Control average of 5					113	

Series	VII	VIII	IX	X	XI	XII	XIII
Orange juice before shaking	37,200	8,750	89,400	7,350	9,500	2,800	8,300
Orange juice after shaking	159	163	172	28	99	24	232
Control average of 5					103		

cause of the stimulus is nearly quantitatively removed from orange juice and from navy bean extract by shaking these extracts with Lloyd's reagent. Since Seidell[6] has shown that this reagent re-

[6] Seidell, A., *Public Health Rep.*, 1916, xxxi, 364.

moves the curative factor for polyneuritis these results seem to add to the other evidence that the test is specific for the B vitamine.

Harden and Zilva[7] have maintained that the Lloyd reagent does not remove the C vitamine. We were concerned to prove that, in the reaction we were using, the stimulus was due to the B and not to the C. If the Lloyd-extracted orange juice still contained the C, it was evident that it was not a factor in the test response. To settle this point we extracted orange juice with the reagent, and then with the assistance of Mr. La Mer of Dr. Sherman's staff we used the filtrate on two guinea pigs. One guinea pig with marked scurvy symptoms, was cured in 12 days with the filtrate.

TABLE XI.

Series	I	II	III								
Navy bean extract, Arnold sterilization only	1,927	2,333	1,532	1,976	2,375	4,020	112	537	1,416	2,064	987
Navy bean extract, Arnold plus 3 hrs. autoclave	292	294	1,841	2,617	552	12	896	11	3,019	56	321
Controls	40	90	Average 32.								

Average with Arnold alone, 1,668; Arnold plus autoclave, 1,036 (Series 3).

The other, a normal guinea pig, was placed on a scurvy-producing diet and given doses of the filtrate to see if it was protective. This guinea pig is still normal with no signs of scurvy after 25 days. These tests seem to confirm the view that in the juice shaken with Lloyd's reagent there was still plenty of the C vitamine and that it does not give the yeast stimulus.

In the experiments with the Bachmann test we seemed to have some evidence that the causative factor was heat-labile at autoclave temperature but stable at 100°C. We therefore repeated these tests with the new technique (Table XI). These results seem to show that at 120° a partial destruction of the vitamine takes place and becomes visible in the test if the amount of vitamine present is not above the optimum for growth.

[7] Harden, A., and Zilva, S. S., *Biochem. J.*, 1918, xii, 93.

All authors are agreed that heat plus alkali are destructive of the B vitamine to a marked degree. To test this point and determine whether the cause of our results was alkali-labile, tests were made with results as given in Table XII.

The first test was inconclusive but the succeeding ones seemed to indicate progressive destructive effects with increase in alkali concentration. The objection can be raised that the inhibition was due to the effect of the alkali on the yeast and not on the vitamine, and the results are now being checked by repeating the tests with neutralized mixtures.

TABLE XII.

Series	I	II				
1 cc. navy bean extract plus 1 cc. 0.1 N NaOH	146	52	9	5	48	50
1 cc. navy bean extract plus 0.75 cc. 0.1 N NaOH plus 0.25 cc. H_2O	112	267	27	39	112	
1 cc. navy bean extract plus 0.50 cc. 0.1 N NaOH plus 0.50 cc. H_2O	170	4,372	96	93	136	
1 cc. navy bean extract plus 0.75 cc. 0.1 N NaOH plus 0.25 cc. H_2O	116	5,257	364	266	165	

1 unit of each mixture used in the test.

From the above results it seems fair to conclude that the cause of the stimulation of yeast growths in this test is heat-labile, apparently alkali-labile, removable by Lloyd's reagent, and present in Funk's purified antineuritic vitamine mixture. It may of course be something different from the B vitamine but if so it behaves remarkably like that hypothetical substance.

Some Applications of the Test.

Up to the present time we have devoted little attention to applications of the test. A few results, however, have been obtained that bear on important points.

Through the kindness of Dr. N. R. Blatherwick we were given samples of jugular vein and mammary vein plasma from a pregnant cow. This material was studied to determine whether or not the technique was applicable to blood tests, since, if this were

so, the possibility of its application to the distribution of the substance in the body and in organs follows. The plasmas were poured into 95 per cent alcohol, the alcohol was filtered off and evaporated to dryness, and the residue taken up in 10 cc. of water. We had for our use about 10 cc. of each plasma so that the solutions used were comparable in strength to the original plasma. One unit of these solutions was used in the tests (Table XIII). The blood plasmas give the test and the mammary vein seems markedly richer in the causative agent indicating that the mamma, as would naturally be inferred, is a mobilizing point for the vitamine.

Investigations have been started in the estimation of the vitamine content of food-stuffs. The work of Mendel and Osborne[8] in their feeding experiments with rats has undertaken the classification of these food-stuffs as to their vitamine B content. Such

TABLE XIII.

Series	I	II				III				
Jugular plasma	29	31	44	59	28	46	21	55	30	44
Mammary "	264	344	623	329	908	858	74	36	1,482	106
Controls	58	48	37	59		24				

substances therefore offer particularly good material for tests since the confirmation of their results would go far toward establishing the practical value of the test in this direction. To enter this field it was first necessary to establish a method of application which would detect relatively slight differences in concentration. Until we can produce absolute uniformity in the yeast suspension it becomes necessary to eliminate the variability in the units of yeast used by increasing the number of tests to a range within the statistical field of accuracy. To that end we adopted the following procedure. First, establish by counting of units the probable range of variation in the suspension used. Second make from five to ten tests on each substance tested and in these test results eliminate all zero readings as showing that the unit in that case contained no cells. Average all other readings. Repeat as often as seems necessary to clear up doubtful po-

[8] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 187; xxxix, 29; 1920, xli, 451.

sitions. Since it is possible to complete 100 tests, counts and all, in 3 to 4 days, plotted results by this method should approximate to accurate results. It is this work that we now have under way and the results to date follow.

The materials already tested include extracts of alfalfa, potato, onion, radish, cucumber, carrot, celery, tomato, turnip, and apple. These materials were first dried in a 60°C. oven and then pulverized. 1 gm. of each substance in the dry form was extracted for the same length of time in boiling water. The water extracts were filtered and made up to the same volume. These extracts were then sterilized in the Arnold sterilizer. In our first series we used one unit of the extract in each test and five tests on each extract. The reactions were so great that our counts ran into the tens of thousands and made comparisons uncertain. We therefore diluted each extract with five volumes of water and repeated the tests. These results are presented in Table XIV.

TABLE XIV.

Substances tested in order of potency.	Cell counts.					Average.
Alfalfa	76	23	145	18	352	123
Potato	11	97	38	55	37	48
Celery	65	41	41	16	1,312*	41
Apple	28	41	0	53	37	39
Tomato	18	69	0	21	17	31
Cucumber	26	0	0	39	12	29
Turnip	28	33	19	19	42	28
Radish	18	21	23	12	2	15
Onion	2	31	0	5	21	15
Carrot	7	0	6	17	30	15
Controls	3	5	0	12		7
Count of yeast units	4	2	7	1		4

* Not included in the average as obviously due to presence of clump in yeast unit.

They show that in certain of the substances the variations and differences are too slight to rest the case on this one series but they harmonize rather well with feeding results to date in the cases of alfalfa, potato, and tomato. They are given as illustrative of methodology and not of final conclusion.

SUMMARY.

1. This paper presents experiments conducted with both the Bachmann and Williams technique for vitamine measurement and presents a new technique which seems to eliminate some of the difficulties in manipulation of these two tests.

2. Evidence is presented in regard to the specificity of the test that seems to confirm the view that the cause is the B vitamine and that the presence of the C vitamine does not in any way affect the test.

3. Two applications of the test are given as illustrative of its field of application.